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Effects of sea-dumped chemical warfare on Baltic Sea ecosystem

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Wpływ zatopionej broni chemicznej na ekosystem Morza Bałtyckiego

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Abstract

Sea-dumping was for many years considered as safe disposal of various materials that were hazardous on land. This also applied to the problematic surplus chemical warfare material (CWM) that remained in warehouses both after World War I and World War II. It is estimated that by the end of WWII in 1945 the worldwide stocks of chemical warfare agents (CWAs) reached about 500,000 metric tons. Although back then, from the on-land risk-management perspective, sea-dumping was supposed to be a "consequence-free" and time-efficient utilization of massive quantities of poisonous substances. Nowadays we know that such transfer has only created a new risk. Later on, the underwater chemical warfare (CW) dumpsites were mismanaged and left unmonitored, while the sea-dumping of CWM was not prohibited until the London Convention in 1972. Till this time, from the very first disposal of munitions containing Lewisite (the arsenic-based CWA) "somewhere" between England and the United States by the U.S. forces in the Atlantic Ocean in 1918, hundreds of thousands of tons of CWA had been sea-dumped worldwide. Globally, there are 127 documented chemical munitions dumpsites, however, it is estimated that their number exceeds 300. In theory, those hazardous substances were supposed to be disposed at the designated areas at depths exceeding 4,000 meters. In fact, the economy behind those peace-making operations in Europe resulted in submerging such objects in much shallower, often coastal areas. Furthermore, there are numerous records of sea-dumping operations beginning already on route towards dumpsites. Therefore, a proper management of the problematic sea-dumped CW was impossible from the very beginning of this process. There were, however, some indications backing up this inaccuracy that seemed to diminish any form of urgent precaution. The general low water solubility of CWAs together with their tendency to undergo hydrolysis and oxidation were believed to neutralize the problem in case of any leakage from the submerged containers. The Baltic Sea alone became a dumpsite for at least 40,000 tons of CW containing 15,000 tons of CWAs, mainly – up to 80% – sulfur mustard (HD). Release of those compounds to the environment was also estimated to start no sooner than 70 years from sea-dumping, what usually is far enough for a problem not to be worth consideration. Unfortunately, this belief remains strong among many policymakers and stakeholders who are now in charge of the aquatic environment protection. It was until 2005 when the first scientific research in the Baltic Sea, the MERCW – Modelling of Environmental Risks Related to the Sea-Dumped Chemical Weapons project, confirmed munitions corrosion

and detected the CWA presence in the sediments, what started changing the overall perspective. Nearly a decade later, thanks to the results obtained during CHEMSEA – Chemical Munitions Search and Assessment, MODUM – Towards the Monitoring of Dumped Munitions Threat, DAIMON – Decision Aid for Marine Munitions and NCN PRELUDIUM 2017/27/N/NZ8/02813 projects, I am able to provide sufficient indications that the environmental consequences of long-term munitions mismanagement for the Baltic Sea ecosystem are much more serious and complex than anyone could expect in the mid-XX century [1][2][3]. In presented PhD thesis I have formulated a hypothesis that sea-dumped chemical warfare affects the biotic parts of the Baltic Sea ecosystem. Therefore, in the first step I have performed a *in situ* habitat description in the dumpsite areas [1]. This work was followed by the laboratory estimation of the effects of sulfur mustard [2] and Clark I [3] release into the aquatic environment. To perform such categorization of threats, an appropriate model organism had to be selected [2][3].

The Baltic Sea is a relatively young ecosystem that became highly affected by the anthropogenic pressure. This shallow and brackish *quasi*-inland sea is isolated from the North Sea by the Danish Straits, which limit its water exchange with the World Ocean. As a result, it has a West-East geographical gradient of significantly decreasing salinity. Located in the moderate climate zone, with an abundant human population living in its catchment area, the Baltic Sea has been influenced by the excessive amounts of xenobiotics and nutrients, originating from the on-land use of pesticides and fertilizers. In general, the nutrient overload increases the eutrophication of the whole waterbody, resulting in massive algal blooms that in consequence cause the oxygen deficiency upon decomposition. This especially affects deeper parts of the Baltic Sea, located below the halocline level, where strong vertical stratification additionally limits the mixing of the water column. As a result, there is a long-term and significant dissolved oxygen (DO) depletion in the vast areas of the sea-bottom and in near-bottom waters. It is visible in a form of so-called “benthic deserts”, where the presence of eucaryotic life forms is highly limited. In general, such areas are characterized by very low biodiversity, often being completely uninhabited by macrofauna while seldomly visited by demersal fish.

Baltic Sea CW dumpsite borders perfectly overlap with the areas affected by the close-to-permanent hypoxia and anoxia. Therefore, to identify the potential risks associated with sea-dumped chemical munitions presence on ecosystem, the first objective of my PhD research was to design and perform a detailed habitat description and first site-specific comparison of these dumping grounds [1]. Results collected between 2012 and 2017, that are presented in a form of a wide-range multidisciplinary examination, allowed me to confirm the already disturbed nature of those areas, due to the observed oxygen depletion. In case of the Bornholm, Gotland and Gdańsk Deeps, where the official and unofficial CW disposal took place, the most likely source of DO in the near-bottom layers is the Major Baltic Inflow (MBI), that is a result of a specific meteorological regime that has an quasi-decadal frequency [1]. Whenever it occurs, this phenomenon pushes great amounts of oxygen-rich, saline waters which due to their density follow the bathymetric structure of the Baltic Sea, enriching those areas with the DO [1]. This means that for the majority of the time, the Baltic Sea CW dumpsites are likely to be uninhabited by macrofauna. Nevertheless, during presented studies there was a close-to-constant presence of meiofaunal assemblages in the dumping areas, however, limited to the single taxonomic group – Nematoda [1]. Fortunately for my monitoring programme, in the December of 2014, there was a third largest recorded MBI in history. It turned out that for a short period between March 2015 and March 2016, local biodiversity in the Bornholm Deep flourished, revealing presence of highly oxygen-dependent meiofaunal taxa including, among the others, Polychaeta and Harpacticoida [1]. In the meantime, the ongoing underwater visual inspection in the vicinity of the dumped objects using Remotely Operated Vehicles (ROV) revealed the active presence of *Monoporeia* sp. (Amphipoda) representing mobile macrofauna, [1]. Regardless from the DO accessibility, during the lifespan of the whole monitoring campaign I have observed the infrequent presence of individuals of two demersal fish species: the Atlantic Cod (*Gadus morhua*) and the European flounder (*Platichthys flesus*) [1]. This would suggest, that despite being a "benthic desert", under certain circumstances, both lethal and sublethal effects of the CWA exposure could appear on various trophic levels. However, any of applied methods did not allow me to confirm such observations *in situ*.

Since more than 70 years have passed from the dumping operations, at least part of the CW was expected to start leaking, releasing CWAs into the environment. Indeed, from the earliest studies in the MERCW project, there has been a constant detection of CWAs both in the Baltic Sea sediments and in pore water. Advancements in analytical methodology, together with the series of laboratory experiments resulted not only in the identification of intact CWAs in numerous of the collected samples but also led to the discovery of a variety of novel CWA-degradation products. When exposed to the aquatic conditions, CWA may i.e. undergo hydrolysis, oxidation, reduction or polymerisation. Moreover, some of those newly formed compounds might be more soluble than the parent compounds. While the recognized toxicity to humans and terrestrial organisms was one of the principles for the CWA weaponization, there is hardly any available information about their aquatic toxicity. This led to the second and third objective of my PhD thesis, the CWA aquatic acute toxicity assessment [2] and observation of sublethal effects [3] that had to be performed in specialized laboratories, under controlled experiment conditions.

Proper wide-range toxicity studies require application of numerous analytical methods and selection of an appropriate organism. Furthermore, the legal and safety issues, due to the requirements of the Organisation for the Prohibition of Chemical Warfare (OPCW), CWA research is limited to the short list of accredited laboratories. All experiments had to be performed in the facilities of Military University of Technology (MUT) [2][3]. This led to the complicated adaptation (2016 – 2018) of those specialized analytical laboratories to obtain their biologically neutral status. Moreover, in the pre-screening phase of the studies, we have also had to perform CWA hydrolyzation and solubility experiments prior to the actual studies [2]. Furthermore, since there were no existing toxicity thresholds assessments for the majority of CWAs, all tests had to be performed using a widely accepted aquatic model species. It was achieved via the series of standardized laboratory experiments using a model freshwater organism – *Daphnia magna* (clone DMB) [2][3]. Performed acute toxicity tests were following the guidelines of the Organisation for Economic Co-operation and Development (OECD) [2]. Such acute toxicity tests are a direct way to determine if the dissolved compounds are very toxic: $EC_{50} < 1 \text{ mg} \times L^{-1}$, toxic: $EC_{50} < 10 \text{ mg} \times L^{-1}$, potentially harmful: $EC_{50} < 100 \text{ mg} \times L^{-1}$ or non-toxic ($EC_{50} > 100 \text{ mg} \times L^{-1}$) to this freshwater organism. For this purpose, sulfur mustard and several of its most commonly detected degradation products were tested. I have proven that HD induces negative effects to exposed organisms, despite its low solubility and, in theory, a rapid hydrolyzation into a non-toxic thiodiglycol with a degradation half-life of 8 min 19 sec at 20°C (at which the experiments were conducted) [2]. Exposure to raw HD had similar effects to the one with HD dissolved in acetone. The *D. magna* 48 hrs EC_{50} acute toxicity threshold is $8.9 \pm 0.5 \text{ mg} \times L^{-1}$, classifying sulfur mustard as toxic in water to *D. magna* [2]. Moreover, at $47.3 \pm 2.8 \text{ mg} \times L^{-1}$ the total and permanent immobilization, thus mortality of tested cohorts occurred already after 15 seconds from the exposure to HD. Therefore, my observations stay in the opposition to the theory that sea-dumping was an environmentally neutral disposal or more accurately, neutralization by the aquatic environment. Furthermore, such scenario did not expect the possibility of the degradation products to be as toxic or even more toxic as the parent compound. Meanwhile, in presented research I have found that two of the tested HD degradation products: 1,2,5-trithiepane (very toxic) and 1,4,5-oxadithiepane (toxic) are 10 × and 4 × more toxic to *D. magna* than the parent compound respectively [2]. Moreover, these toxicity thresholds correspond with the measurements from the pore-water samples collected from the Bornholm Deep sediments [2]. However, while the toxicity thresholds estimation serves as the perfect tool for risk-categorisation it does not necessarily have to be ecologically relevant. Although the exposure to the compound in such concentrations results in the rapid death of a single organism, the scale of the contamination does not necessarily have to induce any significant effects on the whole population. Thus, to assess the potential impact on assemblages and ecosystems, the sublethal effects of chronic toxicity have to be estimated. Third objective of my PhD thesis was to perform the first assessment of the sublethal effects of arsenic-based CWA exposure to the model aquatic organism.

Clark I (diphenylchloroarsine) is one of the most frequent among reported CWAs that are being detected in Baltic Sea sediments. This organoarsenical has been an important component of the Arsine Oil, that served as a thickening additive to the Nazi-Germany winter-type sulfur mustard mixture called Winterlost. Thanks to the presented research it is now known that the sublethal concentrations of Clark I affect the life-histories and population of characteristics of *D. magna* already at the levels of 5 – 10 $\mu g \times L^{-1}$ [3]. This goes along with the results of the preliminary OECD Test No. 211: *Daphnia magna* reproduction test, which indicated Clark I *D. magna* chronic EC_{50} to be at $8.9 \pm 0.5\ \mu g \times L^{-1}$. Particularly important are the observed decrease in size at first reproduction and somatic growth rate, both being the parameters that are widely recognized as crucial for the cladoceran fitness. Moreover, our study examined the effects of Clark I during a relatively short exposure period of 5 days. Thus, the lowest concentration of this phenylarsenical that affects the life-history and population parameters of *D. magna* under chronic exposure could be significantly lower. The estimated amount of the sea-dumped Clark I in the Baltic Sea does not exceed 1,500 tons. If, theoretically this load was equally dissolved in the total volume of the Baltic Sea waters, the resulting concentration would be $0.05\ \mu g \times L^{-1}$. This is only two orders of magnitude lower that the lowest concentration, which in the presented study significantly affected the life-history parameters of *D. magna* [3]. However, due to the bathymetry of the Baltic Sea, water-column stratification and its hydrological regime, it should be considered as a serious hazard for local infauna and whole benthic communities. Indeed, all concentrations at which we have observed the toxic effects of Clark I are significantly lower than its detected quantities in Baltic Sea CW dumpsite sediment and pore-water samples.

The DMB clone, used in both acute and chronic toxicity effects and thresholds estimation [2][3], origins from the Grosser Binnensee lake, that is influenced by the temporal intrusions of the saline waters from the Baltic Sea. Therefore, observed toxic effects could be directly transferrable as i.e. effects to meiofauna assemblages (particularly Harpacticoida), during their reoccurrence as a result of MBI. Except for the exhibition of sublethal effects, there is also a possibility of CWA bioaccumulation in the tissues of filter feeding crustaceans and further propagation to the next generation in the yolk of their eggs. From the perspective of a Crustacean population, few months of habitable conditions can be enough to produce dozens of new generations, however, the presence of CWA in the pore-water may significantly decrease the recolonisation effectiveness or increase the contaminant uptake, which may result in its spreading among the whole Baltic Sea food-web.

For instance, Clark I oxidation product has been detected in the muscle tissues from the 14% of cod (*Gadus morhua*) samples from the Bornholm Deep during the monitoring programs of MODUM and DAIMON projects. The metabolic pathway of Clark I in both the aquatic and terrestrial conditions still remains unknown, however, it is unlikely that such a big number of fish that naturally bioaccumulated this compound had been a result of a direct uptake from the environment. Especially, taking the low solubility in water of the parent compound into account. It is rather a result of biomagnification in the food chain that starts with the compound bonding with detritus in sediments under anoxic conditions. This means that problem of CWA exposure directly affects a whole food web.

In case of fish, a general Fish Disease Index studies can be applied to verify if organisms from the dumpsite area exhibit features of possible contact with CWAs. It is, however, uncertain if the bioaccumulation rate will be higher in the weaker organisms, or the organisms are weaker because of the bioaccumulated amounts of the toxin. This means, there is an urgent need for the new type of CWA-specific biomarkers, i.e. isotopic signatures [3], to obtain more accurate monitoring results in the future assessments.

Presented combination of fieldwork with the laboratory-based approach, focusing on basic studies in the fields of ecology, ecotoxicology and taxonomy provides a broad spectre of novel information around the topic of sea-dumped chemical munitions and their impact on the Baltic Sea ecosystem [1][2][3]. It also means that numerous existing assessments need to be revisited and updated. The biodiversity in the Baltic Sea CW dumpsites is low, however it has been proven, that MBIs can result in recolonisation of sediments by both meiofauna and macrofauna, at least at the Bornholm Deep area [1]. In the following part, the detailed laboratory research provided range sets of important information about the basic physical-chemical characteristics of CWA behaviour in the aquatic conditions [2][3]. Fortunately, by following the standardized OECD toxicity tests, presented results are applicable for any further risk-categorisations and risk-assessments regarding the Baltic Sea CW dumpsites [2][3]. Sea-dumping of munitions should no longer be considered as their neutralization [2][3]. This is beside the fact, that the model organism applied in my studies originates from the freshwater ecosystem [2][3]. However, for a more accurate estimations for the Baltic Sea ecosystem, further research focusing on the toxicity of CWAs on marine species is recommended. It is worth underlining, that during presented studies, due to safety precautions, I did not test any of the HD transformation products of HD polymerisation [2]. Those compounds are known to be significantly more water-soluble, yet still induce similar adverse effects on human health as infamous “mustard gas” and were found at the surface layer of the HD lumps caught in the Bornholm Deep area by fishing nets.

It must be emphasized, that the continuous and pioneering research happening in the Baltic Sea CW dumpsites makes them globally the most recognized dumping grounds for munitions. Despite being a severely disturbed ecosystem, due to the impact of human activity at its catchment, all observed problems at the Baltic Sea area can be directly applicable to any CW dumpsites, especially those located in the areas where human impact is not that significant. My results, together with similar findings from the recent decade, are a solid indication, that beside scientific recognition, after 70 – 100 years, the subject of sea-dumped CW finally deserves proper management and urgent precaution.

Streszczenie

Zatapianie w morzu było przez wieki popularnym sposobem utylizowania przedmiotów i substancji, których przechowywanie na lądzie było niebezpieczne lub problematyczne. Tak też postąpiono z olbrzymim arsenałem broni chemicznej, niewykorzystanym w działaniach wojennych XX wieku. Szacuje się, że pod sam koniec II Wojny Światowej w zasobach wojsk na całym świecie znajdywało się nawet 500 000 ton Bojowych Środków Trujących (BST). W tej sytuacji, zwłaszcza ze względu na niską świadomość ekologiczną w owych czasach, masowe zatapianie wydawało się być doskonałym rozwiązaniem problemu, jaki zasoby te stanowiłyby na lądzie. Dzisiaj, w obliczu wciąż pojawiających się nowych doniesień naukowych, rozwiniętej wiedzy o ekologii i funkcjonowaniu systemów morskich, należy się na poważnie zastanowić, czy proces ten nadal może być traktowany jako utylizacja, czy tylko spowodował przesunięcie problemu w czasie i przestrzeni. Już od pierwszego zatopienia przez wojska amerykańskie broni chemicznej (zawierającej arsenowy BST - Luizyt) „gdzieś między Wielką Brytanią, a Stanami Zjednoczonymi” w roku 1918, operacje te były prowadzone niestarannie, aż do ich zakazania przez „Konwencję Londyńską” w 1972 roku. Wykonywano je bez odpowiedniego raportowania oraz przestrzegania przyjętych założeń. Z początku zakładano zatapianie broni chemicznej na obszarach o głębokościach przekraczających 4 000 metrów, tak aby zminimalizować potencjalnie negatywne efekty dla stref przybrzeżnych. Jednak w obliczu kosztów tych operacji, a także, żeby ograniczyć zagrożenie dla załóg statków wykonujących masowy transport i zatapianie, z czasem zaczęto je wykonywać na znacznie mniejszych głębokościach. Szacuje się, że na całym świecie znajduje się od 127 do 300 podwodnych składowisk broni chemicznej w morzach i oceanach. Co gorsza, istnieją potwierdzone doniesienia, o rozpoczynaniu przez marynarzy procesu jej zatapiania jeszcze w momencie płynięcia z portów na obszary specjalnie do tego celu przeznaczone. Skutkuje to odczuwalnym w dzisiejszych badaniach naukowych brakiem precyzyjnych lokalizacji zatopionych obiektów. Dodatkowo, rozproszenie tych niebezpiecznych obiektów uległo w międzyczasie znacznemu zwiększeniu, poprzez oddziaływanie na nie procesów naturalnych i działalności rybaków. Z drugiej strony, ówczesna wiedza, pozwalała zakładać, że zatapianie skutecznie rozwiązuje i neutralizuje problem BST. Wynikało to z poszlakowych informacji o słabej rozpuszczalności w wodzie tych, w większości, ciał stałych i cieczy. Wiadomo było także, że część z nich ulega gwałtownej hydrolizie. Wiedza ta pochodziła z eksperymentów

przeprowadzanych w laboratoriach chemicznych, w których związki te były syntetyzowane na potrzeby działań ofensywnych, ale też do opracowywania metod przeciwdziałania skutkom ataku. W samym tylko Morzu Bałtyckim zatopiono przynajmniej 40 000 ton broni chemicznej zawierającej nawet 15 000 ton Bojowych Środków Trujących. Większość, bo aż 80% stanowił iperyt siarkowy w różnych postaciach i mieszaninach. Pozostałych 20% stanowiły BST zawierające głównie arsen i fosfor. Przewidywano, że metalowe pojemniki, pociski i bomby zaczną przeciekać w wyniku korozji nie wcześniej niż po 70 latach. Problem ten wydawał się więc na tyle odległy, że nie brano pod uwagę ewentualnych skutków ekologicznych. Jedynym zagrożeniem, o którym się ewentualnie wspominało, była możliwość bezpośredniego kontaktu rybaków z tymi substancjami w wyniku wyłowienia zbiorników w sieciach. Do dnia dzisiejszego istnieje z resztą silne przekonanie, że obecność zatopionej broni chemicznej nie stanowi wielkiego zagrożenia dla środowiska, a jeśli już, to tylko w lokalnej skali. Świadomość potencjalnego ekologicznego zagrożenia zaczęła się stopniowo zmieniać dopiero po 2005 roku, dzięki wynikom pierwszego bałtyckiego projektu badawczego dotyczącego zatopionej broni chemicznej. W projekcie MERCW – Modelling of Environmental Risks Related to the Sea-Dumped Chemical Weapons potwierdzona została obecność broni chemicznej na dnie Głębi Bornholmskiej, zarówno w postaci prawie nienaruszonych, jak i niemal całkowicie skorodowanych obiektów. Wykryto także po raz pierwszy BST w próbkach osadów z miejsc zatapiania. Prezentowana praca doktorska jest efektem kontynuacji tych pionierskich badań.

Przyjąłem hipotezę, iż zatopiona broń chemiczna implikuje negatywne skutki na ożywione elementy ekosystemu Morza Bałtyckiego. Aby ją zweryfikować, w pierwszej kolejności opisałem biotop oraz stwierdziłem jaki jest stan ilościowy oraz jakościowy biocenozy na obszarach zatapiania [1]. Następnie przeprowadziłem laboratoryjną kategoryzację zagrożeń płynących z przedostawania się do środowiska iperytu siarkowego [2] oraz arsenowego BST – Clark'a I [3] na odpowiednio dobranym organizmie modelowym [2][3]. Prezentowane przeze mnie wyniki, pochodzące z projektów CHEMSEA – Chemical Munitions Search and Assessment, MODUM – Towards the Monitoring of Dumped Munitions Threat, DAIMON – Decision Aid for Marine Munitions oraz NCN PRELUDIUM 2017/27/N/NZ8/02813, rzucają nowe światło na ten „schowany" ponad 70 lat temu w głębinach mórz problem [1][2][3].

Morze Bałtyckie jest stosunkowo młodym, płytkim akwenem o niskim zasoleniu oraz bardzo ograniczonej wymianie wód ze wszechoceanem. Od Morza Północnego oddzielają je płytkie Cieśniny Duńskie, w wyniku czego obserwowany jest tu silny Zachodnio-Wschodni gradient malejącego zasolenia. Morze Bałtyckie znajduje się w strefie klimatu umiarkowanego, a jego zlewnia jest jedną z najgęściej zaludnionych (85 milionów ludzi). Zatopienie broni nastąpiło zatem na obszarze młodego ekosystemu o prostych sieciach troficznych, który równolegle poddany został bardzo silnej antropopresji w wyniku dostarczania do niego olbrzymich ilości nawozów i ksenobiotyków skutkujących wysoką eutrofizacją i zanieczyszczeniem wody. Eutrofizacja objawia się wzmożoną produkcją pierwotną, rezultatem której jest nadprodukcja materii organicznej, która opadając na dno przyspiesza zużywanie rozpuszczonego tlenu w procesie jej rozkładu. Problem ten dotyka w szczególności głębin znajdujących się poniżej halokliny, gdzie powstają tak zwane „pustynie tlenowe", w których obecność organizmów eukariotycznych jest mocno limitowana. Głębie Morza Bałtyckiego charakteryzuje niska bioróżnorodność, często obserwowany całkowity brak makrozoobentosu oraz tylko sporadyczne pojawianie się w nich ryb przydennych. Oficjalne granice składowisk broni chemicznej w Morzu Bałtyckim pokrywają się z granicami „pustyń tlenowych", co sugerować mogłoby zerową bądź znikomą, aktywność biologiczną w sąsiedztwie zatopionych obiektów [1].

Nadrzędnym celem mojego doktoratu była weryfikacja tej tezy poprzez zaprojektowanie i przeprowadzenie pierwszego na tych obszarach szczegółowego i całościowego opisu habitatów [1]. Prezentowane wyniki, pochodzące z szeroko zakrojonych badań o multidyscyplinarnym charakterze, pozwalają w pierwszej kolejności na potwierdzenie faktu deficytu tlenowego w rejonach Głębi Bornholmskiej, Głębi Gotlandzkiej i Głębi Gdańskiej [1]. Dzięki monitoringowi, który prowadziłem w latach 2012 – 2017, stwierdziłem, iż w typowych warunkach składowiska broni chemicznej na obszarach głębi bałtyckich charakteryzują się całkowitym brakiem występowania organizmów makrozoobentosowych [1]. Z uwagi na silną stratyfikację Morza Bałtyckiego przyjmuje się, że jedynym znaczącym źródłem tlenu w tych częściach tego akwenu mogą być wlewy z Morza Północnego [1]. Zjawisko to, obserwowane przeciętnie raz na dekadę, polega na napływie dużych ilości dobrze natlenionych i wysoko zasolonych, gęstych wód w wyniku panowania odpowiednich i długotrwałych warunków meteorologicznych [1]. Masy te, poruszając się zgodnie z batymetrią akwenu Morza Bałtyckiego dostarczają duże ilości rozpuszczonego tlenu poniżej poziomu halokliny [1]. W grudniu 2014 roku miał miejsce trzeci co do wielkości zarejestrowany wlew z Morza Północnego. W rejonie Głębi Bornholmskiej jego efekty zaczęto obserwować dopiero po marcu 2015. Szacuje się, iż dostarczony przez wlew rozpuszczony tlen cząsteczkowy, mógł utrzymać się na tym obszarze przez okres 8 miesięcy. Okres ten wystarczył, aby w marcu 2016 roku udało mi się zaobserwować dowód rekolonizacji składowisk broni chemicznej, zarówno na poziomie meiofauny, jak i makrofauny [1]. W typowych latach deficytu tlenowego w osadach i wodach przydennych na obszarach wszystkich przebadanych składowisk, jedynymi przedstawicielami meiofauny, były nicienie (Nematoda) [1]. Dostarczenie przez wlew dużych ilości dobrze natlenionej wody zaowocowało w 2016 roku znajdywaniem w próbkach osadu z Głębi Bornholmskiej taksonów, które charakteryzuje duża wrażliwość na zaniki tlenu, m.in. przedstawicieli wieloszczetów (Polychaeta) oraz widłonogów (Harpacticoida) [1]. Kolejnym skutkiem wlewu było pojawienie się w tym samym rejonie i czasie osobników *Monoporeia sp*., czyli niewidzianych we wcześniejszych latach monitoringu przedstawicieli makrofauny [1]. W marcu 2016 roku uzyskałem wizualne potwierdzenie aktywności przedstawicieli tego rodzaju w bezpośrednim sąsiedztwie zatopionych obiektów pochodzenia militarnego przy pomocy systemu kamer zdalnie sterowanego robota typu ROV – Remotely Operated Vehicle. Obie te obserwacje oznaczają, że podwodne składowiska broni chemicznej w Morzu Bałtyckim, w wyniku okresowego zjawiska jakim jest wlew wód z Morza Północnego, ulegają tymczasowej rekolonizacji przez organizmy bentosowe [1]. Niezależnie od warunków tlenowych, przez cały okres monitoringu, przy pomocy systemu kamer ROV, udało mi się również stwierdzić sporadyczną obecność dwóch gatunków ryb: dorsza atlantyckiego (*Gadus morhua*) oraz storni (*Platichthys flesus*) w sąsiedztwie zatopionych obiektów [1]. Niestety, synergia negatywnego oddziaływania na organizmy morskie potencjalnych efektów ekspozycji na BST z deficytem tlenu uniemożliwia oszacowanie i oddzielenie poszczególnych składowych zastosowanymi metodami *in situ*.

Od zatopienia w Morzu Bałtyckim broni chemicznej minęło 70 lat. Czas ten, od początku traktowany był za moment, w którym przynajmniej część z zatopionych obiektów zacznie korodować, przy okazji uwalniając potencjalnie toksyczne związki do środowiska. Sukcesywna i rokrocznie raportowana w ostatniej dekadzie detekcja różnego rodzaju BST w osadach z miejsc zatapiania nie jest więc zaskoczeniem. O ile toksyczne właściwości BST dla ludzi i organizmów lądowych zostały poznane w wyniku przeprowadzenia wielu jawnych i niejawnych eksperymentów w laboratoriach wojskowych, zakładano, że wiele z tych związków ulegnie w środowisku wodnym neutralizacji. BST mogą ulegać w środowisku morskim różnego rodzaju transformacjom: od hydrolizy, przez utlenianie lub redukcję, po polimeryzację. Wyraźną lukę w stanie naszej wiedzy stanowił zatem brak informacji na temat ich toksyczności w wodzie, a jej uzupełnienie stało się jednym z celów mojej pracy [2][3]. Co roku dochodzi także do detekcji znanych, ale i tych nowo odkrywanych dla nauki produktów ich degradacji. Ich toksyczność w środowisku wodnym również była nie w pełni poznana. Próby odpowiedzenia na ten problem badawczy wymagały jednak przeprowadzenia badań w kontrolowanych, laboratoryjnych warunkach [2][3].

Choć określenie czy związek jest toksyczny w wodzie z pozoru wydawać się może nieskomplikowanym zadaniem, w rzeczywistości poprawnie zaprojektowane oszacowanie wartości progów toksyczności wymaga zaangażowania wielu technik analitycznych oraz dobrania odpowiedniego organizmu modelowego. Dodatkowo, charakter badanych związków oraz formalne obostrzenia dostępu do BST wynikające z „Konwencji o zakazie broni chemicznej" wymagały pierwszego w historii i długofalowego dostosowania specjalnych pomieszczeń i procedur bezpieczeństwa na akredytowanym w Organizacji ds. Zakazu Broni Chemicznej (OPCW) Wydziale Nowych Technologii i Chemii Wojskowej Akademii Technicznej w Warszawie do pracy z żywymi organizmami. Metodą prób i błędów, w latach 2016 – 2018 udało się nam wspólnie wyeliminować wpływ wszystkich czynników zewnętrznych chemicznie czystego, choć nieneutralnego biologicznie laboratorium, które w początkowym etapie praktycznie uniemożliwiały przeprowadzanie zaprojektowanych eksperymentów. Ponadto, aby uzyskać jak najbardziej naukowo poprawne i akceptowalne wyniki zdecydowałem się na, nieintuicyjne z perspektywy biologa morza, wykorzystanie do tych celów słodkowodnego przedstawiciela wioślarek – *Daphnia magna* [2][3]. Po pierwsze, hodowla organizmów rozradzających się w wyniku partenogenezy pozwala na uzyskanie kohort o znikomym zróżnicowaniu genetycznym. W rezultacie, ograniczony zostaje wpływ cech osobniczych na efekty ekspozycji na badany związek, przy równoległej produkcji dużej liczby jednowiekowych organizmów. Dodatkowo, *D. magna* jest organizmem modelowym wykorzystywanym powszechnie w monitoringu środowiska, przez co dostępne są w literaturze instrukcje standardowych testów toksyczności, a uzyskane w nich wyniki pozwalają na kategoryzację zagrożenia i porównywanie efektów badanego związku z dostępną bazą toksyczności innych substancji. Dzięki tym przygotowaniom możliwe stało się przeprowadzenie pierwszych w historii szeroko zakrojonych badań wpływu BST na organizmy wodne [2][3], które do tej pory ograniczały się głównie do prac na niektórych produktach ich degradacji. Równolegle przeprowadzony został szereg testów dotyczących oszacowania maksymalnych rozpuszczalności w wodzie części z siarkowych i arsenowych

BST oraz wybranych produktów ich degradacji, w tym także oszacowania tempa hydrolizy niektórych z tych związków [2].

Parametry toksyczności ostrej, czyli „effect concentration" - EC_{50}, w których efekty immobilizacji *D. magna* obserwuje się u 50% osobników z badanej grupy organizmów w danym stężeniu, wyznaczyłem na podstawie wyników uzyskanych w standardowych testach Organizacji Współpracy Gospodarczej i Rozwoju (OECD). Dzięki temu, możliwa była kategoryzacja zagrożenia płynącego z ekspozycji przedstawicieli tego gatunku na konkretny związek chemiczny. W zależności od stężenia, w którym obserwowany jest efekt immobilizacji *D. magna*, uważa się związek za silnie toksyczny ($EC_{50} < 1$ mg × L^{-1}), toksyczny ($EC_{50} < 10$ mg × L^{-1}), potencjalnie szkodliwy ($EC_{50} < 100$ mg × L^{-1}) bądź nietoksyczny ($EC_{50} > 100$ mg × L^{-1}). W ramach mojej pracy doktorskiej przeprowadziłem eksperymenty na iperycie siarkowym oraz sześciu produktach jego degradacji i hydrolizy [2]. Udało mi się udowodnić, że mimo swoich hydrofobowych właściwości, chemicznie rozpuszczony w acetonie iperyt jest toksyczny dla *D. magna*, z *48h* EC_{50} na poziomie 8.9 ± 0.5 mg × L^{-1} [2]. Ponadto, w stężeniu 47.3 ± 2.8 mg × L^{-1}, związek ten powodował permanentną immobilizację całej badanej kohorty już po 15 sekundach od ekspozycji. Rezultatem tej immobilizacji była 100% śmiertelność badanych organizmów [2]. Miało to miejsce, mimo niemal natychmiastowej hydrolizie tego związku, którego okres półtrwania w temperaturze 20°C to 8 minut i 19 sekund [2]. Niemal identyczny rezultat miało dodanie możliwie najmniejszej kropli iperytu siarkowego [2]. Tu jednak hydrofobowe właściwości iperytu powodowały uformowanie przez niego trwałej kulistej formy, która opadła na dno. Śmiertelne efekty wywołał najprawdopodobniej kontakt z jej powierzchnią, emitowanymi produktami degradacji bądź uwalnianiem się czystego iperytu do medium. O ile bezpośredni produkt hydrolizy iperytu siarkowego, czyli tiodiglikol jest nietoksyczny, o tyle dwa z przebadanych przeze mnie produktów jego degradacji wykazały znacznie silniejsze toksyczne właściwości od związku macierzystego. 1,2,5-tritiepan (bardzo toksyczny) oraz 1,4,5-oxaditiepan (toksyczny) okazały się być kolejno 10 × i 4 × bardziej toksyczne od iperytu siarkowego [2]. Co ważniejsze, stwierdziłem, iż wartości progów toksyczności obu tych związków są zbliżone do wykrywanych stężeń w osadach i wodach porowych osadów Morza Bałtyckiego [2]. Proces zatopienia BST w morzach nie może być zatem uznany za ich neutralizację ani tym bardziej nie jest neutralny dla biocenozy.

O ile wartości progów toksyczności ostrej są doskonałym narzędziem służącym kategoryzacji zagrożeń, o tyle nie służą one szczegółowej analizie ich oddziaływania na ekosystem. To, co dla pojedynczego organizmu może być śmiertelną dawką, z punktu widzenia populacji i w zależności od wielkości skażenia może być nieistotne. Nie dotyczy to jednak wartości progów toksyczności chronicznej. Parametr ten pozwala na obserwację subletalnych efektów ekspozycji na daną substancję. Efektów, które w dłuższej perspektywie, mogą wpłynąć na kondycję całej populacji, a które, w kontekście BST, nie zostały jeszcze opisane w dostępnej naukowej literaturze. Kolejnym celem mojej pracy doktorskiej było zatem przeprowadzenie serii testów z wykorzystaniem *D. magna*, tym razem w kontekście dłuższej niż 48 godzin ekspozycji na działanie difenylochloroarsyny – Clark'a I.

Clark I jest jednym z najczęściej wykrywanych BST w osadach Morza Bałtyckiego, związek ten był składnikiem tzw. Oleju Arsenowego, który dodawany był w nazistowskich Niemczech do „zimowego" iperytu siarkowego typu Winterlost. Dostępne raporty szacują jego zatopioną w tym akwenie ilość na około 1 500 ton. W wyniku przeprowadzenia 21-dniowego testu OECD, w którym mierzyłem zmianę dzietności *D. magna* w efekcie ekspozycji na Clark'a I, udało mi się oszacować chroniczne EC_{50} tego związku na poziomie $8.9 \pm 0.5\ \mu g \times L^{-1}$. Dzięki temu, przy współpracy ze specjalnie stworzonym i kierowanym przeze mnie międzyinstytucjonalnym zespołem, możliwe były późniejsze badania dotyczące wpływu BST na historie życia trzech klonów *D. magna* [3]. Chroniczne efekty arsenopochodnych BST na bezkręgowce wodne, a w szczególności na kluczowe gildie, takie jak wioslarki planktonowe, nie były dotychczas w ogóle zbadane. W prezentowanej pracy stwierdzamy, że subletalne stężenia Clark'a I powodują zmiany najważniejszych dla wioślarek planktonowych parametrów historii życia (płodności, wielkości ciała, tempa wzrostu osobnika) oraz tempa wzrostu populacji [3]. W przyszłych ocenach zagrożenia wywieranego przez arsenopochodne BST na populacje organizmów zasiedlających skażone akweny należy więc uwzględnić te efekty. Odkryciem jest fakt, że po ekspozycji na difenylochloroarsynę skład izotopów azotu w ciele wioślarek uległ zmianie [3]. Dotychczas nie zbadano nigdy wpływu zanieczyszczenia na sygnatury izotopowe organizmów. Stwierdziliśmy ponadto, że wioślarki różnią się interklonalnie pod względem wrażliwości na toksyczne działanie Clark I, co może sprzyjać wykształceniu adaptacji pozwalających na utrzymywanie się różnych populacji w skażonych akwenach.

Jeśli teoretycznie cały zatopiony ładunek Clark'a I zdołałby się równomiernie wymieszać w całej wodzie Morza Bałtyckiego, jego stężenie wynosiłoby $0,05\ \mu g \times L^{-1}$. To wartość o dwa rzędy wielkości mniejsza od najniższego stężenia, w którym zaobserwowaliśmy istotne statystycznie efekty ekspozycji wszystkich klonów badanego gatunku rozwielitek na difenylochloroarsynę. Jednak w rzeczywistości, ze względu na niską rozpuszczalność tego związku, ale i stratyfikację kolumny wody, jak i batymetrię Morza Bałtyckiego, obserwujemy lokalne i dużo wyższe stężenia w osadach i wodach porowych. Oznaczać to może lokalne występowanie stężeń wyższych niż chroniczne *D. magna* EC_{50}, które wpływać mogą na tempo i skuteczność rekolonizacji tych obszarów przez meiofaunę i makrofaunę podczas kolejnych wlewów. Twierdzenie to opieram o wykorzystanie przeze mnie w badaniach specyficznego klonu *D. magna*, pochodzącego z niemieckiego jeziora Grosser Binnensee [2][3]. W jeziorze tym odnotowuje się intruzje wód z Morza Bałtyckiego, przez co klon DMB posiada pewne przystosowania do okresowego przebywania w wyższym zasoleniu. Oznacza to, że uzyskane wyniki, mogą korespondować z potencjalną odpowiedzią gatunków typowo morskich, zwłaszcza skorupiaków wchodzących w skład meiofauny.

Wszystkie prezentowane badania, nie wykluczają także możliwości bioakumulowania się BST lub ich produktów degradacji w tkankach badanych organizmów. Niestety, uzyskiwana w ten sposób biomasa nie była wystarczająca do zaobserwowania tego zjawiska u *D. magna*, mimo obserwacji nawet 20%-owego zaniku Clark'a I w niektórych stężeniach podczas badań laboratoryjnych. Związek ten został za to niedawno wykryty w śladowych ilościach w próbkach tkanki mięśniowej 14% osobników dorsza (*Gadus morhua*), złowionych trałem pelagicznym na obszarze Głębi Bornholmskiej w ramach programu monitoringowego ICES oraz projektów MODUM oraz DAIMON. Nadal praktycznie nic nie wiadomo o szlaku metabolicznym difenylochloroarsyny, zarówno u przedstawicieli lądowej jak i morskiej fauny, nieznana więc jest droga jego bioakumulacji w Morzu Bałtyckim. Trudno jednak przypuszczać, by ten słabo rozpuszczalny związek uległ odłożeniu się u tak dużej ilości osobników wyłącznie w wyniku bezpośredniego wniknięcia ze środowiska. Najprawdopodobniej jest to wynik biomagnifikacji, która byłaby efektem bioakumulacji tego związku, przez organizmy z niższych poziomów troficznych, być może w łańcuchu detrytusowym, który swój początek miał w skażonej materii organicznej w osadach. Niezależnie od tego, wykrycie bioakumulowania się produktów degradacji w tkankach mobilnych drapieżników, znajdujących się niemal na szczycie piramidy troficznej Morza Bałtyckiego, obala argument o wyłącznie lokalnym oddziaływaniu BST na ekosystem.

Poza bezpośrednią analizą chemiczną obecności i efektów tych związków w badanych organizmach wodnych, nie istnieją jeszcze biomarkery specyficzne dla ekspozycji na BST. O ile w przypadku ryb, jednym ze sposobów szacowania efektów oddziaływania tych związków na osobniki przebywające na obszarach zatapiania broni chemicznej jest tzw. Fish Disease Index (FDI), nie tłumaczy on związku przyczynowo-skutkowego. Możliwe jest na przykład, w wyniku konkurencji wewnątrzgatunkowej, przebywanie w bezpośrednim sąsiedztwie zatopionych obiektów i skażonych osadów osobników słabszych i już wcześniej chorych. Osłabienie mogłoby wtedy sprzyjać szybszej bioakumulacji tych związków. Z tej przyczyny, jedno z prezentowanych przeze mnie odkryć, dotyczące sygnatur izotopowych [3], może być w przyszłości zalążkiem opracowania nowego, a zarazem tak potrzebnego wskaźnika ekspozycji organizmów na to konkretne zanieczyszczenie środowiska.

Prezentowana w tej rozprawie kombinacja prac terenowych i badań laboratoryjnych skupiających się na badaniach podstawowych w zakresie problematyki zatopionej broni chemicznej przedstawia szereg nowych informacji, niedostępnych wcześniej dla świata nauki [1][2][3]. Jest ona także częścią szeroko zakrojonej aktualizacji stanu wiedzy, za którą odpowiedzialni są naukowcy pracujący w projektach CHEMSEA, MODUM i DAIMON. Oznacza to, że wiele wniosków ze wcześniejszych założeń i opracowań sprzed 2005 roku wymaga rewizji. Dzięki mojej pracy udało się w holistyczny sposób opisać habitaty na obszarach zatapiania broni chemicznej oraz potwierdzić stałą obecność nicieni wchodzących w skład meiofauny [1]. Stwierdziłem także okresową rekolonizację tych obszarów przez przedstawicieli makrozoobentosu oraz wzrost bioróżnorodności meiobentosu w wyniku oddziaływania wlewu z Morza Północnego [1]. Z kolei w części laboratoryjnej badań został obalony argument o neutralizacji BST przez zatapianie [2][3]. W rzeczywistości iperyt okazał się być toksyczny w środowisku wodnym dla organizmu modelowego jakim jest *D. magna* [2]. To samo dotyczy dwóch produktów jego degradacji oraz arsenowego BST – Clark'a I [2][3]. Część z nowopowstających w wyniku transformacji w środowisku wodnym produktów degradacji posiadać może znacznie bardziej toksyczne właściwości od macierzystych BST, przy równoczesnej większej rozpuszczalności w wodzie [2]. Niepokojąca jest również obserwacja istotnych statystycznie zmian w historii życia rozwielitek w bardzo niskich stężeń arsenowego BST – Clark'a I [3]. Ponadto, opisane przeze mnie progi toksyczności części z przebadanych substancji są niższe od ich stężeń wykrywanych w osadach Morza Bałtyckiego [2][3].

Niestety, wiele konsekwencji zatapiania BST w morzach i oceanach jest nadal dla świata nauki niejasnych, więc ciężko o jednoznaczną analizę problemu. Przygotowania do laboratoryjnych testów toksyczności udowodniły nieścisłości w dostępnej literaturze na temat tak podstawowej właściwości jaką jest wartość maksymalnej rozpuszczalności danego BST w wodzie [2]. Dodatkowo, podczas badań dotyczących toksyczności iperytu siarkowego, z uwagi na niebezpieczeństwo pracy z niektórymi związkami, nie szacowaliśmy progów toksyczności produktów jego polimeryzacji [2]. Związki te są dużo bardziej rozpuszczalne w wodzie od związku macierzystego oraz wiadomo, że posiadają zbliżone do niego właściwości parzące. Dodatkowo, były one wykrywane na powierzchni brył iperytu siarkowego wyłowionych przez rybaków na obszarze Morza Bałtyckiego. Warto także podkreślić, że trzy opisywane przeze mnie składowiska stanowią światowy poligon badawczy dla problematyki zatopionej broni chemicznej. Oznacza to, że znaczna większość publikowanych w ostatniej dekadzie przełomowych opracowań i wyników pochodzi raptem z 1 – 2,4% ze wszystkich podwodnych składowisk. W dodatku, prezentowane i przytaczane obserwacje miały miejsce na obszarze akwenu już wcześniej znajdującego się pod silną antropopresją. Prezentowane wyniki mogą się zatem przekładać na potencjalne obserwacje na obszarach poza jego granicami. Prawdopodobnie są też na świecie niezbadane jeszcze składowiska broni chemicznej, w których degradacja środowiska w wyniku jej zatopienia byłaby dużo bardziej wyraźna. Osobiście uważam więc, że okres 70 – 100 lat od zatapiania to najwyższa pora, by problem ten został w końcu dostrzeżony, a instytucje odpowiedzialne za ochronę mórz i oceanów podjęły próbę przeciwdziałania jego konsekwencjom.

List of Research Papers:

[1] *Deep sea habitats in the chemical warfare dumping areas of the Baltic Sea.* **Czub M.**, Kotwicki L., Lang T., Sanderson H., Klusek Z., Grabowski M., Szubska M., Jakacki J., Andrzejewski J., Rak D., Bełdowski J. Science of The Total Environment 2018, Vol. 616-617, 1485-1497

[2] *Acute aquatic toxicity of sulfur mustard and its degradation products to Daphnia magna.* **Czub M.**, Nawała J., Popiel S., Dziedzic D., Brzeziński T., Maszczyk P., Sanderson H., Fabisiak J., Bełdowski J., Kotwicki L. Marine Environmental Research 2020, Vol. 161, https://doi.org/10.1016/j.marenvres.2020.105077

[3] *The effects of chemical warfare agent Clark I on the life histories and stable isotopes composition of Daphnia magna.* Brzeziński T., **Czub M.**, Nawała J., Gordon D., Dziedzic D., Dawidziuk B., Popiel S., Maszczyk P. Environmental Pollution 2020, Vol. 266 (3), https://doi.org/10.1016/j.envpol.2020.115142

CRediT authorship contribution	**1**	**2**	**3**
Conceptualization	**MC**, TL, HS, JB, LK	**MC**, TB, PM, HS, JB, LK	TB, **MC**, SP, PM
Data curation	**MC**	**MC**, JN, SP, DD, TB, PM, HS, LK	
Formal analysis	**MC**, JB	**MC**, JN, SP, JF, JB, LK	TB,
Funding acquisition	JB, LK	**MC**, TB, PM, JF, JB, LK	
Investigation	**MC**, MG, JJ, MS, DR, ZK, JB	**MC**, JN, SP, DD, TB, PM	TB, **MC**, JN, DG, DD, BD, PM
Methodology	**MC**, ZK, JJ, JB, LK	**MC**, JN, SP, TB, HS, JB	TB, SP, PM
Software	**MC,** MG, JJ, ZK	**MC**, SP, HS, JB, LK	
Validation	**MC**, JB, JJ	**MC**, JN, SP, DD, TB, PM, HS, JF, JB, LK	
Visualization	**MC**, MG, JA, DR, ZK	**MC**, JN, SP, PM, HS, JF	
Project administration	JB	**MC**, JB, LK	
Resources	JB, LK	**MC**, TB, JF, JB, LK	
Supervision	TL, HS, JB, LK	**MC**, TB, PM, HS, JF, JB, LK	TB, PM
Writing - original draft	**MC**	**MC**, JN, DD, TB, PM, JF	TB
Writing - review & editing	**MC**, TL, HS, MG, MS, DR, ZK, JB, JJ, LK	**MC**, SP, TB, HS, JB, LK	**MC**, JN, DG, DD, SP, PM

MC – Michał Czub, TB – Tomasz Brzeziński, PM – Piotr Maszczyk, HS – Hans Sanderson, JB – Jacek Bełdowski, DD – Daniel Dziedzic, JN – Jakub Nawała, SP – Stanisław Popiel, LK – Lech Kotwicki, JF – Jacek Fabisiak, DG – Diana Gordon, BD – Barbara Dawidziuk, MG -Miłosz Grabowski, TL - Thomas Lang, JJ – Jaromir Jakacki, JA – Jan Andrzejewski, DR – Daniel Rak, MS – Marta Szubska, ZK – Zygmunt Klusek

Research Paper no. 1

Deep sea habitats in the chemical warfare dumping areas of the Baltic Sea.

Michał Czub, Lech Kotwicki, Thomas Lang, Hans Sanderson, Zygmunt Klusek, Miłosz Grabowski, Marta Szubska, Jaromir Jakacki, Jan Andrzejewski, Daniel Rak, Jacek Bełdowski

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Deep sea habitats in the chemical warfare dumping areas of the Baltic Sea

Michał Czub [a],*, Lech Kotwicki [a], Thomas Lang [b], Hans Sanderson [c], Zygmunt Klusek [a], Miłosz Grabowski [a], Marta Szubska [a], Jaromir Jakacki [a], Jan Andrzejewski [a], Daniel Rak [a], Jacek Bełdowski [a]

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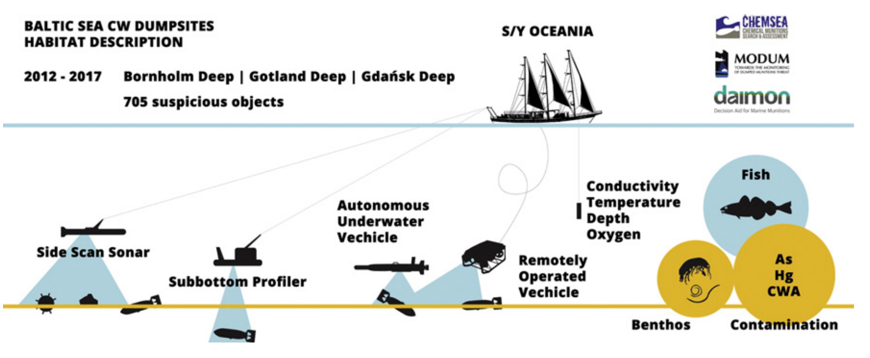
HIGHLIGHTS

- Habitats in three Baltic Sea Chemical Warfare dumpsites were described as Deep-Sea Muddy Sands.
- All investigated basins belong to so-called "benthic-deserts".
- Multidisciplinary studies have been performed.
- Dumpsites were investigated before, during and after a Major Baltic Inflow event.
- Temporary Return of benthic macrofauna was observed in one basin.

GRAPHICAL ABSTRACT



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ABSTRACT

The Baltic Sea is a severely disturbed marine ecosystem that has previously been used as a dumping ground for Chemical Warfare Agents (CW). The presence of unexploded underwater ordnance is an additional risk factor for offshore activities and an environmental risk for the natural resources of the sea. In this paper, the focus is on descriptions of the marine habitat based on the observations arising from studies linked to the CHEMSEA, MODUM and DAIMON projects. Investigated areas of Bornholm, Gotland and Gdańsk Deeps are similarly affected by the Baltic Sea eutrophication, however, at depths greater than 70 m several differences in local hydrological regimes and pore-water heavy metal concentrations between those basins were observed. During the lifespan of presented studies, we were able to observe the effects of Major Baltic Inflow, that started in December 2014, on local biota and their habitats, especially in the Bornholm Deep area. Reappearance of several meiofauna taxa and one macrofauna specimen was observed approximately one year after this phenomenon, however it's ecological effects already disappeared in March 2017. According to our findings and to the EUNIS Habitat Classification, the three reviewed areas should be characterized as Deep Sea Muddy Sands, while the presence of suspicious bomb-like objects both beneath and on top of the sediments confirms their CW dumpsite status.

1. Introduction

Underwater unexploded ordnance is now recognized to pose serious threats to the marine environment and its users (Greenberg et al., 2016). This is a very different attitude compared to that of the mid-20th century, when sea-dumping was a common practice (Elmgren, 2001). Oceans and seas were believed to be a limitless and safe place to dispose of unused munitions until the London Convention in 1972. Sea-dumping operations took place worldwide at all depths and both conventional and chemical warfare (CW) materials were submerged (Edwards and Bełdowski, 2016; Greenberg et al., 2016). The combination of

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administrative, political and military issues left the dumpsites unmonitored for many decades (Knobloch et al., 2013). Ongoing natural processes together with offshore activities such as fishery have significantly expanded the areas of potential exposure (Sanderson et al., 2009). Not only do the munitions contain explosives and toxic agents, but they are also considered as a source of heavy metals and metalloids, mainly arsenic, to the environment (Bełdowski et al., 2016b). Every dumping area is characterized by a different set of environmental parameters and various types or quantities of disposed warfare material. Site-specific risk assessments should be performed on a case-by-case basis.

Covering a surface area of 415,000 km^2, the Baltic Sea is relatively shallow, with an average depth of 52 m. It was formed during the last glaciation and the present level of salinity stabilized 2000 years ago, becoming one of the largest brackish ecosystems in the world. Salinity values in the Baltic Sea range between 1 and 20 PSU, with an average of 7 PSU. This young ecosystem provides multiple natural services for a large human population living in the catchment area, while being highly sensitive to many forms of human impact (Elmgren, 2001).

Numerous reports and reviews including Knobloch et al. (2013), Bełdowski et al. (2016a) and Greenberg et al. (2016) indicate that soon after the end of World War II, the Baltic Sea began to be used as a dumpsite for at least 40,000 tons of chemical warfare agents (CWA). The toxic loads accounted for up to 15,000 tons, 80% of it which was mustard gas (Knobloch et al., 2013). It was during the pioneering MERCW project - Modelling of Ecological Risks Related to Sea-Dumped Chemical Weapons (http://mercw.org) - when the existence of submerged munitions in the Bornholm Deep area was confirmed. It has resulted in first scientific observations of several completely corroded casings (Missiaen et al., 2010) and allowed researchers to indicate potential site-specific hazards related to the CWA presence in the sediments (Sanderson et al., 2010). In subsequent CHEMSEA project - Chemical Munitions, Search and Assessment (www.chemsea.eu) - both CWA and their degradation products were detected in pore-water and sediments detected in roughly 40% of cases (Bełdowski et al., 2016a) thanks to the newly-developed methods (Popiel et al., 2014). Also, first indications of adverse effects on Baltic Fish were reported (Bełdowski et al., 2016a). Follow-on work under the MODUM project - Towards the Monitoring of Dumped Munitions Threat (http://www.iopan.gda.pl/MODUM) - focused on creation of monitoring network and assessing the environmental risk of CWAs in the Baltic Sea which included testing the toxicity of selected degradation products (Christensen et al. 2016) and Weight of Evidence (WoE) analyses (Bełdowski et al., 2017). Interdisciplinary research is nowadays continued in the DAIMON project - Decision Aid for Marine Munition (http://www.daimonproject.com) - with the aim to develop the risk assessment algorithms and the decision support system.

1.1. Study area

Little Belt, Bornholm and Gotland Deeps are recognized to be the most important, officially designated CWA dumpsite areas in the Baltic Sea. The CW dumping area commonly referred to as the 'primary dumpsite', is located in the Bornholm Deep centred on a point with surface coordinates 55°20′N, 15°37′E. Its northern part is currently marked on sea charts as 'larger explosives dumping ground'. Sea-dumping operations in the Gotland Deep took place between May and September 1947, when approximately 2000 tons of CW materiel consisting of 1000 tons of CWA were dumped. On the other hand, studies performed in CHEMSEA project confirmed the existence of an unofficial dumpsite in the Gdańsk Deep (Bełdowski et al., 2016a). The suspicion about CWA presence in Gdańsk Deep arose after two incidents, the first with a mustard gas bomb recovered by a fishing trawler and a second with similar bomb being washed ashore on the Hel Peninsula in 1954 (Szarejko and Namieśnik, 2009). The CWA presence was finally verified by pore-water (Bełdowski et al., 2016a). The total volume of dumped conventional munitions in Gdańsk Deep until 1954 was approx. 60 tons, however the load of CWA is still unknown (Knobloch et al., 2013).

Although the loads of sea-dumped CWA are believed to pose a possible threat to the Baltic Sea ecosystem, there is already an existing environmental degradation linked with nutrient overload that caused reduction of dissolved oxygen (DO) concentrations in bottom waters and creation of a "benthic deserts" below the halocline (Diaz and Rosenberg, 2008). Water stagnation has negative impacts on marine ecosystems, especially in accumulation basins, since states of hypoxia and anoxia not only negatively influence organisms (Vaquer-Sunyer and Duarte, 2008), but also their habitats (Conley et al., 2009).

During more than 100 years of Baltic Sea research (Elmgren, 2001), the areas of Bornholm, Gdańsk and Gotland Deeps have been widely studied. Among studies of singular basins, several comparisons have been performed (i.e. Vallius and Kuzendorf, 2001, Christoffersen et al., 2007). The aim of this study was a site-specific identification of the ecological status of CW dumpsites located in those three deeps of the Baltic Sea. A detailed description of factors governing the conditions in the dumpsite areas can be a useful tool for researchers representing various scientific fields. The presented set of environmental and modelling data relies on recent findings and is designed to serve as background information for researchers and stakeholders engaged with the issue.

2. Materials and methods

Presented habitat characterization is based on the location of the CWA dumpsites in Baltic Proper, exceeding depths of 70 m (Fig. 1). Datasets were collected during the CHEMSEA, MODUM and DAIMON – Decision Aid for Marine Munitions research projects. Three deep-sea Baltic CW dumpsites were investigated during 10 expeditions of S/Y Oceania, R/V Walther Herwig III and R/V Nord 3 between 2012 and 2017 (Table 1). While the CHEMSEA project focused on locating suspicious looking objects, in both MODUM and DAIMON projects those stations where CWA were detected have been revisited.

2.1. Depth, salinity and dissolved oxygen profiles of water column

The CTD (Conductivity, Temperature, Depth) probe SeaBird 49, additionally equipped with an oxygen sensor was used during all cruises. Measurements were performed at every sampling station. The accuracies of the conductivity and pressure sensors were 0.005 $\mathrm{mS \cdot cm^{-1}}$ and 0.1% of the full-scale range, respectively. The conductivity sensor is calibrated annually by the manufacturer to ensure accuracy. The profiles of DO concentration were obtained with the Rinko-I sensor. The accuracy of the sensor was ± 2% (1 Atm, 25 °C) with a resolution of 0.01 to 0.4% (2 to 8 $\mu g \cdot L^{-1}$).

2.2. Water exchange and bottom currents modelling

Baltic Sea water exchange forecasts and suitable representations of actual bottom currents are available thanks to employment of the POP - Parallel Ocean Program (Smith and Gent, 2004). For bottom currents modelling, the hindcast was based upon a 20-years simulation period. The POP model is widely used and refers to global and regional models and was successfully applied for the Baltic Sea. It has a horizontal resolution of 2 km and 66 vertical levels, 50 of which are 5 m in depth.

2.3. Acoustic sediment characterization, sea bottom and sub bottom mapping

Two types of sonars were used to collect acoustic data. The EdgeTech DF-1000 towed sonar was used for preliminary sea bottom mapping,

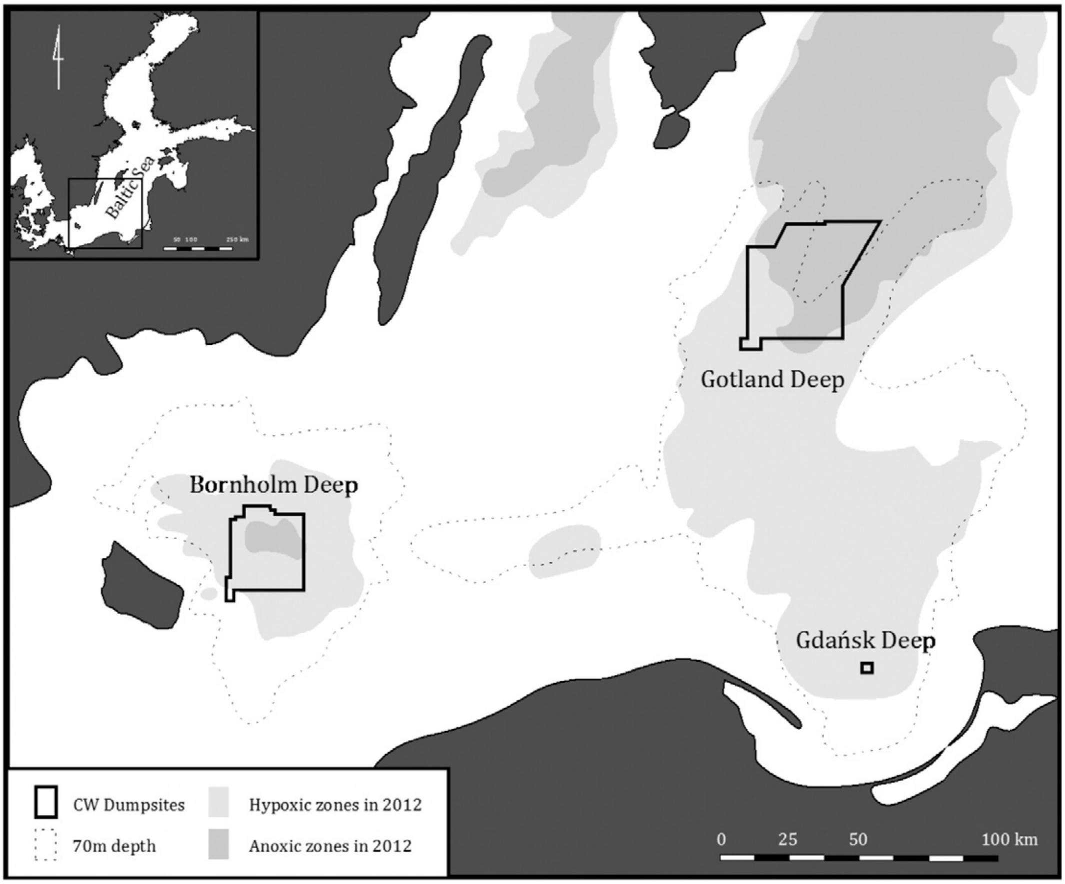

Fig. 1. Locations of deep-sea Chemical Warfare (CW) dumpsites in Baltic Sea area. Anoxic and hypoxic zones in 2012 are redrawn from Carstensen et al. (2014).

performing data acquisition at two resolutions: standard – 100 ± 10 kHz and high resolution – 400 ± 20 kHz, with towing speed of 3 knots (kn), and height above the bottom ranging between 10 and 15 m. Detailed seabed imaging was performed by means of the Ocean Server's AUV IVER-2 with hull-mounted Klein L-3 UUV – 3500, dual frequency side scan sonar (455 kHz and 900 kHz), working at a speed of 2.5 kn at a height ranging from 2.5 to 10 m from the bottom. Collected sonar data served as an input for post processing in HYPACK 2013 software. During the investigation of Gdańsk Deep in September 2015, information about subsurface sediment layers became available thanks to 30 surveys performed by EdgeTech SB3200 XS Sub-Bottom profiler. Additionally, around 200 launches of the Saab Falcon ROV, provided visual information for sea bottom recognition.

2.4. Sediment sampling and analyses

Samples for sediment analyses were collected by means of Van Veen grab and Box Corer at distances <100 m from the suspicious objects. The top 5-cm sediment layer was preserved for two-step grain size analysis. The coarse fraction was separated by passing the sediment through sieves with varying mesh diameters: 2000 μm, 1000 μm, 500 μm, 250 μm, 125 μm, and each fraction was weighed. The fraction smaller than 125 μm was left for subsequent laser measurements performed with MALVERN Mastersizer 2000. Results were later calculated in GRADISTAT 4.0. The organic matter (OM) content of the sediment was calculated based upon the loss on ignition (LOI), after heating the samples at 480 °C for 8 h.

Table 1
Sampling campaigns for sea-dumped Chemical Warfare, including types of collected data.

Year	Area	Project	Vessel	Sediment				CW Detection				Water column
				Type	Metal.	Meio	Macro	SSS	AUV	SUB	ROV	CTDO
2012	*Gotland Deep*	CHEMSEA	OCE	+++	+++	+	+	+			+	+
2012	*Bornholm Deep*	CHEMSEA	WH	+++	+++	+	+					+++
2012	*Gdańsk Deep*	CHEMSEA	OCE	+++	+++	+	+	+			+	+
2014	*Bornholm Deep*	MODUM	OCE	+++	+++			+			+	+
2014	*Gt/Gd Deeps*	MODUM	OCE	+++	+++	+++		+	+		+	+++
2015	*Bornholm Deep*	MODUM	OCE	+	+++	+++		+	+		+	+++
2015	*Gdańsk Deep*	MODUM	OCE/NOR	+	+++	+++		+	+	+++	+	+
2016	*Bornholm Deep*	MODUM	OCE	+	+++	+++		+	+++		+	+
2016	*Gdańsk Deep*	MODUM/DAIMON	OCE	+	+++			+	+++		+++	+
2017	*Bornholm Deep*	DAIMON	OCE	+	+++	+++		+	+++		+++	+

+++ - data presented in this paper; + - data collected during expeditions.
OCE – S/Y Oceania, WH – R/V Walter Herwig III, NOR – Nord 3.
Type – samples for sediment grain size analyses; Metal. – samples for metalloids and heavy metals concentrations; Meio – samples for taxonomical analyses of meiofauna; Macro – samples for taxonomical analyses of macrofauna; SSS – Side-Scan Sonar surveying; AUV – Autonomous Underwater Vehicle surveying; SUB – Sub-bottom profiler surveying; ROV – missions of Remotely Operated Vehicle; CTDO – Conductivity, Temperature, Depth and Oxygen profiling of water.

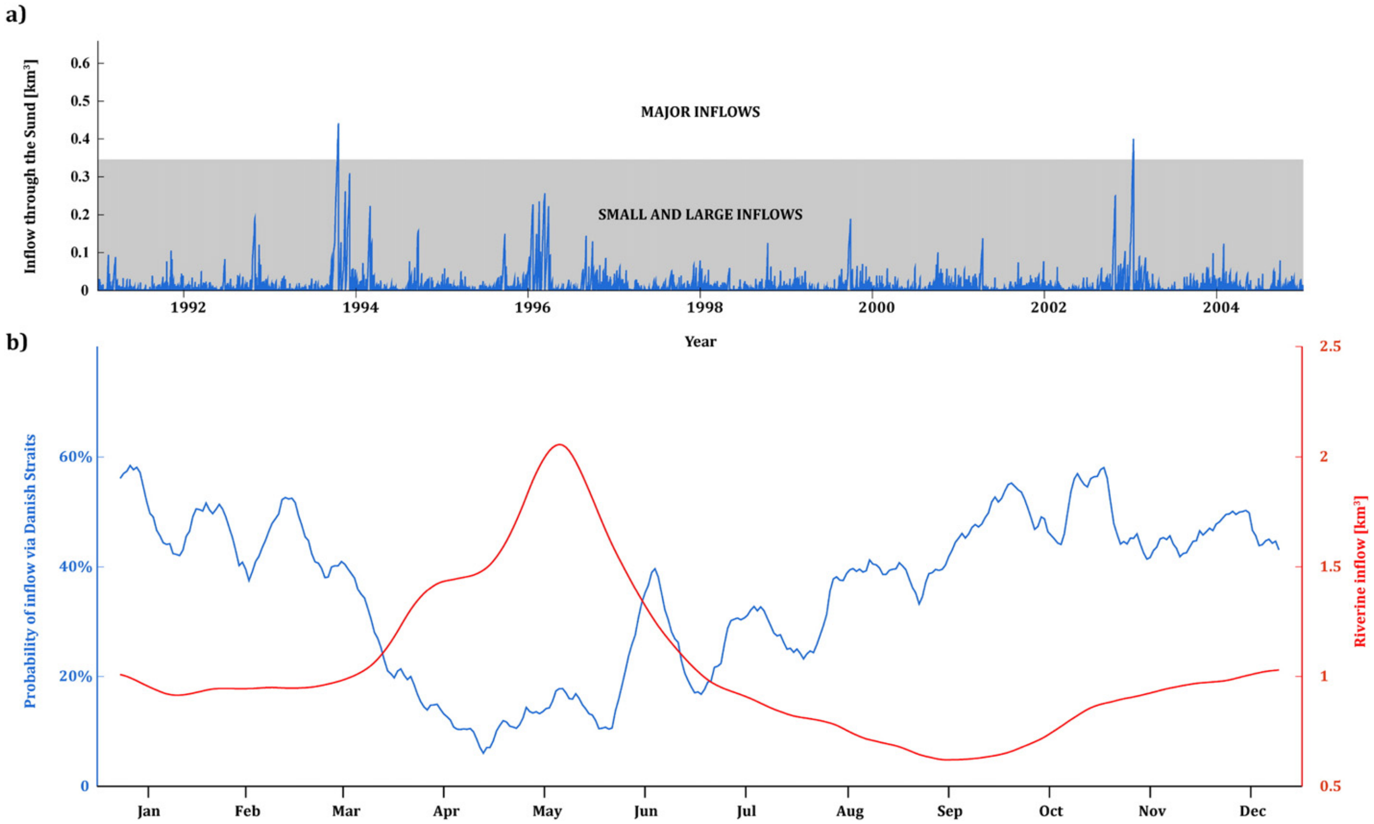

Fig. 2. Inflows of saline waters (a) from North Sea through the Sund strait since 1991 to 2005. The exchange of waters is driven by complexity of factors, however, the probability of inflows increases during autumn and winter, when the riverine inflow is the smallest.

Sediment samples for total arsenic were prepared as in Loska and Wiechuła (2006) by dry digestion for further analysis on hydride generation Atomic Absorption Spectrophotometry (HG-AAS) (Bełdowski et al., 2016b). After digestion, other samples for the concentrations of lead, zinc, iron and manganese were measured with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a Perkin Elmer ELAN 9000. Mercury concentrations were measured by Atomic Absorption Spectrometry using an AMA-254 spectrometer. After wet digestion in acids, sub-samples for the analysis of lead, zinc, iron and manganese concentrations were measured with ICP-MS detector (Perkin Elmer ELAN 9000) combined with seaFAST pre-concentration mode (CF-IDA) containing Iminodiacetic acid immobilized on a vinylbenzyl copolymer was used for measurements of lead and cadmium concentrations.

2.5. Meiofauna sampling and analyses

Samples for taxonomical analyses of benthic meiofauna were collected by means of Van Veen grab, Box Corer or ROV at distances <50 m from the suspicious objects. The top 5-cm sediment layer was subsampled by 10 cm^2 Plexiglas cores and preserved in 10% formalin solution. Organisms were extracted using standard method (Burgess, 2001), by a 10-minute long tripled centrifuging at 1900 RPM in 1,2 $g \cdot mL^{-1}$ LUDOX solution. Samples were later stained using Rose Bengal and analysed under stereoscopic microscopes.

3. Results and discussion

3.1. Pelagic zone

Conditions in bottom waters of the Baltic Proper are controlled by inflows of saline water from the North Sea through the shallow Danish Straits. While most of the exchange is driven by sea level difference between Kattegat and the Baltic Proper only the so-called Major Baltic Inflows (MBI) have a direct impact on sediments and vertical mixing of the whole water column (Carstensen et al., 2014). MBI are characterized by an exceptionally high volume, salinity levels and long duration allowing those dense inflows to penetrate water column down below halocline of the Baltic Proper.

The MBI occur only around ten times per century (Fig. 2a) and are most likely to happen during winter and autumn (Fig. 2b). The MBI from December 2014 was the first large-scale water exchange between the Baltic and North Seas in a decade, bringing approximately 198 km^3 of dense and oxygen-rich water (Morholz et al., 2015). In February/March 2015 MBI effects have been observed in the Bornholm Deep (Fig. 3). As expected, inflow waters appeared below the halocline and were characterized by an exceptionally high salinity, 21 PSU, and relatively homogenous temperature. Observed salinity levels of the freshly

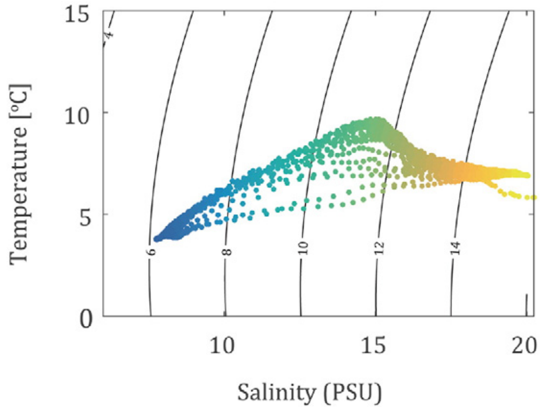

Fig. 3. TS plot based on the data collected in February 2015 from the Bornholm Deep areas exceeding 70 m.

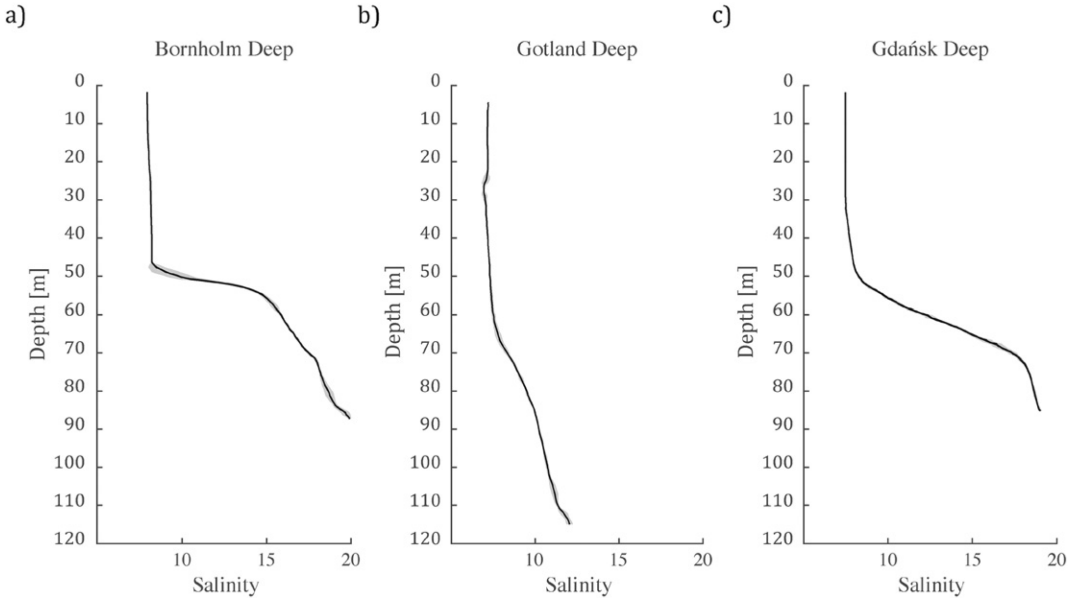

Fig. 4. Mean salinity profiles measured in three investigated dumpsites. The profiles are based on 5 cases at each station: a) Bornholm Deep – February 2015 (55°14.3′N, 15°51.5′E), b) Gotland Deep – September 2015 (56°01.5′N, 18°46.1′E) and c) Gdańsk Deep - October 2015 (55°13.4′N, 15°54.1′E).

transported water were 3 PSU higher than multiyear mean values for bottom waters at Bornholm Deep region (Rak and Wieczorek, 2012).

Among all investigated sites, the dumpsite area of Gotland Deep was the deepest, with depths ranging from 103 to 122 m. Depths at Bornholm Basin varied from 92 to 101, and in Gdańsk Deep from 95 to 108 m. The bathymetries of three investigated basins are therefore slightly different and their depths significantly exceed the average depth of the Baltic Sea.

While the salinity in surface layers was similar at each region investigated with levels around 7 PSU, the deep layers varied greatly with the

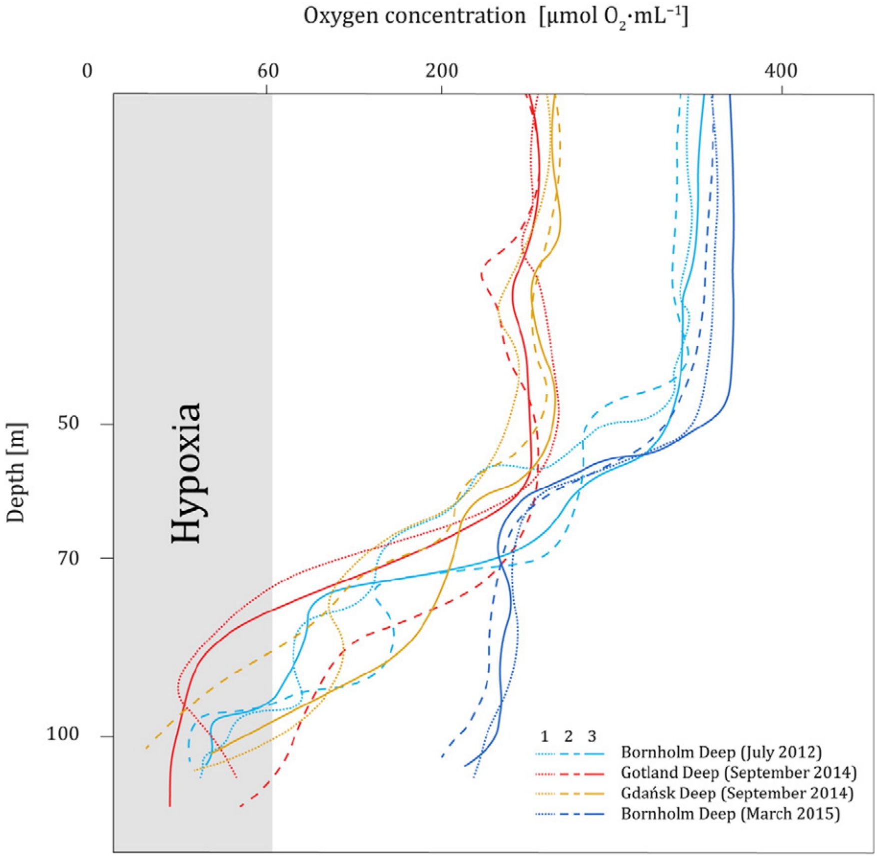

Fig. 5. Dissolved oxygen concentrations in water column at investigated dumpsites: Bornholm Deep in May 2012 (1. 55°12.0′N, 15°42.5′E; 2. 55°21.3′N, 15°48.2′E; 3. 55°20.5N, 15°37.2′E) in March 2015 (1. 55°18.6′N, 15°37.6′E; 2. 55°21.6′N, 15°30.9′E; 3. 55°21.6′N, 15°37.8′E), Gotland Deep in September 2014 (1. 56°01.6′N, 18°46.1′E; 2. 56°00.0′N, 18°46.8′E; 3. 56°01.9′N, 18°45.2′E) and Gdańsk Deep (1. 54°50.5′N, 19°11.0′E; 2. 54°45.2′N, 19°09.9′N Profiles end at the sea bottom. At all measurements from before 2015, dissolved oxygen conditions in bottom waters were extremely poor resulting in severe hypoxia.

difference between Bornholm and Gotland Deep of approximately 6 PSU (Fig. 4). The salinity of the bottom layers decreases with distance from the Danish Straits and is a result of the connection with Atlantic Ocean. At investigated stations, the halocline depths varied from 50 m in Bornholm Deep to 70 m in the Gotland Deep (Fig. 4). In the Baltic Proper it usually forms at approximately 70 m (Carstensen et al.,

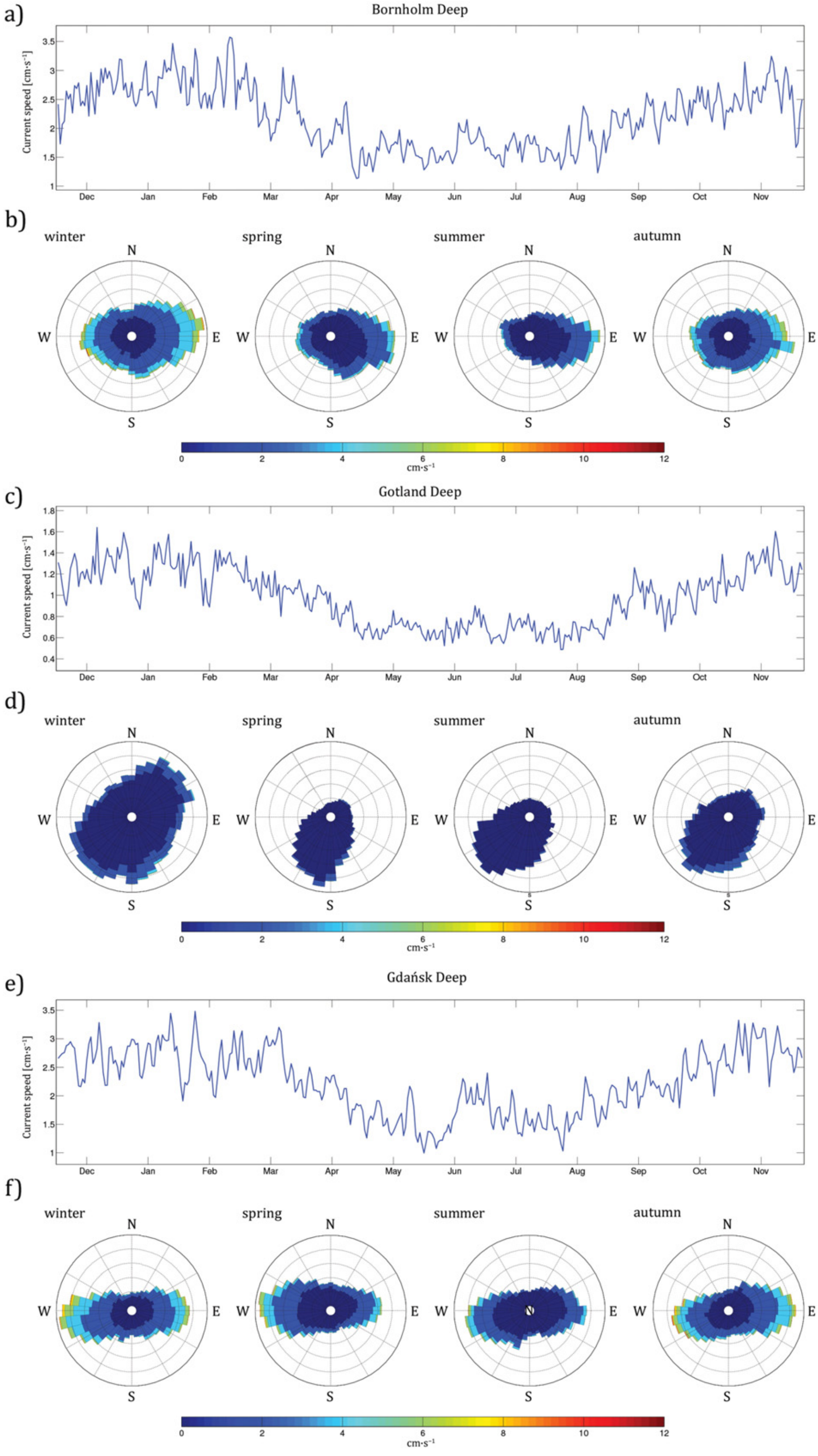

Fig. 6. Mean annual cycle (a, c, e) and current roses (b, d, f) for seasonally averaged near-bottom current speeds: winter, spring, summer and autumn, at three stations: Bornholm Deep (a, b), Gotland Deep (c, d) and Gdańsk Deep (e, f). Modelling has been performed for stations located in Bornholm Deep at 55°19.6′N, 15°38.1′E, in Gotland Deep at 56°01.6′N, 18°46.1′E and in Gdańsk Deep at 54°44.8′N, 19°46.2′E.

2014). Strong stratification of the water column limits vertical mixing, causing hypoxic and anoxic conditions in benthic habitats disturbed by high eutrophication. Prior to the MBI of 2014, DO concentrations in bottom waters at all investigated dumpsites did not exceed 60 $\mu mol \cdot L^{-1}$ (Fig. 5) which is, in part, why MBIs are so crucial for Baltic marine organisms and benthic ecosystems.

Although currents can ventilate bottom waters, under specific conditions they may also act as a dispersal agent for CWA (Bulczak et al., 2016). Seasonal variability of near-bottom currents at investigated areas is represented in Fig. 6. Each plot represents the mean annual cycle of current velocity, while rose diagrams characterize the radial distribution of near-bottom current speed. Those diagrams show the frequency distribution of current velocity binned into classes of current directions. The radial length of each spoke around the circle represents the percentage of time that current flows in a specific direction. Different colours at each spoke represent relative shares of different current speed classes.

Current velocities are similar in the Gdańsk Deep and the Bornholm Deep, and less than half as strong in the Gotland Deep. The mean current speeds in the Gdańsk, Bornholm and Gotland Deeps are 2.13 $cm \cdot s^{-1}$, 2.14 $cm \cdot s^{-1}$ and 0.9 $cm \cdot s^{-1}$, respectively and are characterized by a strong seasonal variability. In general, increased near-bottom current speeds are observed from autumn to early spring (Fig. 6a, c, e). This is likely caused by stronger winds and more frequent storms occurring in the Baltic Sea region in this period. Overall, those areas are low-dynamic sedimentation basins where only occasional, above-mean-value current velocities exceed the 4 $cm \cdot s^{-1}$ sediment resuspension threshold (Bulczak et al., 2016).

The current rose for the Bornholm Deep (Fig. 6b) reveals that in summer the frequency of eastward flow is about 45% (in the direction between 45 and 135°). In this current direction, the frequency of velocities in the range of 0–2 $cm \cdot s^{-1}$ is about 26%, in the range of 2–4 $cm \cdot s^{-1}$ is about 13%, and above 4 $cm \cdot s^{-1}$ is 3%, respectively. A graphical presentation is designed in a way that all directions of bottom currents meet in the centre of the chart, with their beginnings at the edge of the rose. In summer the eastward near-bottom currents are observed about 3 times more often than currents from other directions. Baltic Sea near-bottom currents are mostly topographically steered as their directions are dictated by local bathygraphic features of the sea bottom, thus the prevailing currents in Bornholm Deep are directed inward the Baltic Sea. Seasonal variability observed at this station is likely driven by storms that occur most often in spring and autumn. A similar situation is observed in the Gotland Deep (Fig. 6d). The annual cycle is a result of storms that disturb the mean southwestward current in autumn and winter. In the Gdańsk Deep, a strong annual cycle of the current velocity is observed, while current directions do not change significantly on a seasonal scale (Fig. 6f) what can be explained by the location of the station - the deepest part of the Gdańsk Deep, where the influence of storms is negligible. At each station, stronger winter currents are also driven by inflows from the North Sea. Probability of inflow is the highest in the winter period and larger amounts of water enter the Baltic Sea during winter months (Matthäus and Franck, 1992; Fischer and Matthäus, 1996).

3.2. Benthic zone

Benthic habitats are defined as structural parts of the environment that attract organisms and serve as a centre of biological activity (Peters and Cross, 1992). This definition includes the variety of sediment types as well as the co-occurring structures. At each investigated site, highly-detailed acoustic maps of the sea bottom were created for possible target recognition. Acoustic methods are a very effective, non-invasive tool for seafloor classification (Anderson et al., 2008). In general, all three investigated regions are flat areas covered by soft sediment (Fig. 7). Acoustic mapping results correspond with grain-size laboratory analyses of sediments (Table 2). GRADISTAT results indicate that surface sediments from investigated stations belonged to the sandy muds textural group in Bornholm Basin and to the muddy sands group in both Gotland and Gdańsk Basins. The top 5 cm of sediments from all investigated areas were highly liquefied, with mean water content ranging from 76 to 89%.

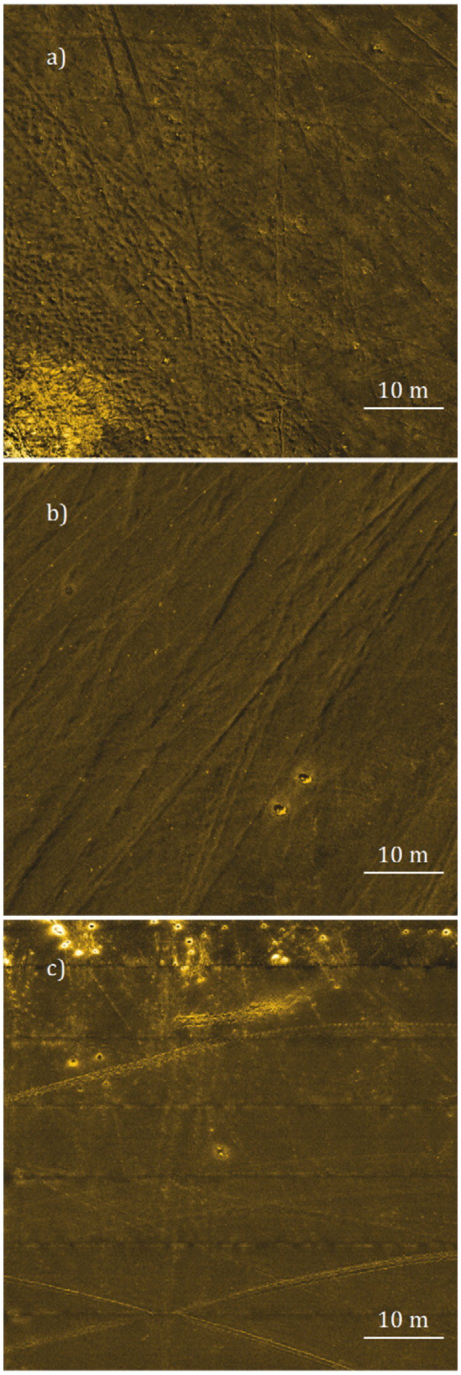

Fig. 7. Results of acoustic sea bottom mapping performed by IVER-2 post-processed in HYPACK 2013 from a) Bornholm Deep – 55°19.0′N, 19°09.7′E, b) Gotland Deep – 56°01.4′N, 18°46.1′E and c) Gdańsk Deep – 54°45.3′N, 15°37.7′E. Dark colours represent soft sediments, while light reflexions originate from various debris laying on top of sediment: a) parts of a shipwreck, b) sea-mines, c) scattered metallic objects. Trawl marks are visible as continuous lines on all images.

The most actual Sub-bottom profiler surveys in the Gdańsk Deep (Bełdowski et al., 2017) correspond with the previous findings from the Bornholm Deep where large fraction of bomb-like objects was observed beneath the soft sediments surface (Missiaen et al., 2010). It also detected large gas pockmarks at similar depths in the sediment.

Table 2
Sediment properties and Arsenic (As) and heavy metals: Mercury (Hg), Cadmium (Cd), Lead (Pb), Zinc (Zn), Iron (Fe) and Manganese (Mn) concentrations at investigated CW dumpsites.

Area	Mean depth [m]	Textural group	<0.063 [%]	OM [%]	H_2O [%]		Metalloids and heavy metals					
							As [$\mu g \cdot g^{-1}$]	Hg [$ng \cdot g^{-1}$]	Pb [$\mu g \cdot g^{-1}$]	Zn [$\mu g \cdot g^{-1}$]	Fe [$mg \cdot g^{-1}$]	Mn [$\mu g \cdot g^{-1}$]
Bornholm Deep	97	Sandy muds	84.7	15	76	Min.	12.5	4.9	16.9	38.5	14.79	614.6
						Max.	22.9	211.1	51.7	237.0	49.92	4328.4
						Mean	17.2	56.6	33.4	**193.4**	43.06	2238.7
						Ref.	*22*	*80*	*43*	*110*		
Gotland Deep	115	Muddy sands	77.7	11	74	Min.	3.7	11.9	8.3	61.1	12.96	147.1
						Max.	23.2	289.9	142.2	275.6	76.13	2663.8
						Mean	13.7	58.4	29.5	**161.0**	40.38	564.6
						Ref.	*14*	*80*	*36*	*98*		
Gdańsk Deep	99	Muddy sands	76.9	19	89	Min.	1.1	9.5	6.1	14.4	4.88	122.5
						Max.	22.6	296.8	64.8	231.1	71.67	544.5
						Mean	15.4	**146.0**	36.6	**180.3**	36.18	362.6
						Ref.	*16*	*100*	*54*	*122*		

<0.063 – fine grained sediment fraction; OM – organic matter; H_2O – water content.
Bold values in Metalloids and Heavy Metals concentrations represent mean values higher than in *Ref.* – reference (Uścinowicz, 2011).

Since there are several gas pockmarks described in the Gulf of Gdańsk (Majewski and Klusek, 2014) and Bornholm Basin (Christoffersen et al., 2007), the methane fluxes may act as an avenue for contaminated pore-waters and move CWA degradation products to surficial sediments.

In the analysed samples, mean OM content in sediments was 11% in Gotland Deep, 15% in Bornholm Deep and 19% in Gdańsk Deep (Table 2). Sedimentary OM is a source of nutrients for benthic communities and it may also be a vector for heavy metals as cadmium, zinc and mercury tend to bond with OM (Pearson, 1963; Fitzgerald and Lamborg, 2005; Callender, 2005). Sedimentation rates in the Gdańsk Deep vary from 0.70 ± 0.13 to 3.90 ± 0.31 mm yr^{-1} (Suplińska and Pietrzak-Flis, 2008). According to Hille et al. (2006), the mean linear sedimentation rate for the whole Gotland Deep is 0.93 ± 0.67 mm yr^{-1}, with values ranging from 3 to 5 mm in central areas (Vallius and Kuzendorf, 2001). Sedimentation rates in Bornholm Deep are the lowest among those three areas, with a range from 0.52 ± 0.02 up to 0.82 ± 0.10 mm yr^{-1} (Suplińska and Pietrzak-Flis, 2008).

Although surface sediment layers have similar morphology, the acoustic mapping revealed minor differences in geological features between investigated areas (Fig. 7). These differences are a result of geographical location. In the Bornholm Basin, situated east of the Bornholm Island, the sedimentary supply originates mainly from coastal erosion, with all remaining part originating from suspending material and atmospheric dust (Stryuk et al., 1995). The vicinity of a major source of coastal material explains why western parts of the investigated area were partially covered by small rocks (Fig. 7a). There were hard sediment patches in Gdańsk Deep (Fig. 7c), where munitions burial in the sediment would be impossible. Located in the south-eastern part of Baltic Proper, this basin is heavily influenced by the largest river of the Baltic Sea catchment area. The Vistula River is an important source of OM and fluvial material in the region, responsible for deposition of sands in the southern parts of the Gdańsk Deep (Damrat et al., 2013).

While the 80% of CW disposed in the Baltic Sea were mustard gas loads, the remaining part consisted of arsenic-based blistering agents such as Adamsite, Clark I and II, Chloroacetophenone and killing agent Tabun (Knobloch et al., 2013). Therefore, Vallius and Kuzendorf (2001), in comparison studies of the geochemistry of Bornholm and Gotland Deeps, linked the elevated arsenic concentrations around Bornholm with CW dumping. Mean values of arsenic concentrations measured from 2012 to 2016 during CHEMSEA and MODUM campaigns in Bornholm, Gotland and Gdańsk Deeps were 17.2 $\mu g \cdot g^{-1}$, 13.7 $\mu g \cdot g^{-1}$ and 15.4 $\mu g \cdot g^{-1}$ respectively (Table 2). Slightly elevated values were observed in one sample from Bornholm Deep, and in nearly 30% of samples from Gdańsk and Gotland Deeps. Detected levels are, however, comparable to arsenic biogeochemical background and do not significantly exceed the geochemical background established for Baltic Sea fine sediments (Uścinowicz, 2011). In previous years, Emelyanov et al. (2010) reported arsenic concentrations in Bornholm Deep with mean value of 82 $\mu g \cdot g^{-1}$ (range 21–210 $\mu g \cdot g^{-1}$) with the highest concentration noted in the centre of Bornholm secondary dumpsite. Paka and Spiridonov (2002) reported 18 and 150 $\mu g \cdot g^{-1}$ in sediments collected in years 1997–2001. In Gotland Deep CW dumpsite, the concentrations ranged from 1 to 19 $\mu g \cdot g^{-1}$ with a mean value of 8 $\mu g \cdot g^{-1}$ and the highest noted value in the dumpsite area, spatially decreasing from the dumpsite (Garnaga et al., 2006). Similar research from Paka and Spiridonov (2002) reported values between 18 and 28 $\mu g \cdot g^{-1}$ sediments collected from 1997 to 2001.

Overall observation, from all presented studies, is that arsenic concentrations in surface sediments from Baltic Sea CW dumpsites decrease year after year. However, both Emelyanov et al. (2010) and Paka and Spiridonov (2002) used total X-Ray diffraction (TXRF), which is characterized by low sensitivity. This may have resulted in some extremes, while the methodology used in this study always represents values averaged across a sample aliquot. If the observed trend is real, then either the contaminated sediments are being covered with recent sedimentary material and the peak concentration of arsenic originating from CWA should be observed deeper in the sediment, or the source of pollution is spreading. Sanderson et al. (2010) detected the degradation products of arsenic-based warfare agents in sediments outside of the primary dumpsite. In his studies, Bełdowski et al. (2016b) indicated that behaviour of arsenic in CW dumpsites differs from typical Baltic Sea sediments, where it bonds with OM, iron and manganese oxyhydroxides, thus, reported lower and/or negative values of Pearson correlation coefficients was possibly explained by a local source of arsenic.

Mercury concentrations at investigated areas exceeded geochemical background values in numerous samples (Table 2), which is corresponding with similar research reported by Gębka et al. (2016). This element tends to bind with OM and its elevated concentrations correlate with high contents of fine grained sediment fraction (<0.063 mm). The biogeochemical cycle enables mercury re-emission from sediments depending on REDOX conditions. Also, measured lead concentration values remained within the range of geochemical background values for this element (Table 2). Only in 3 samples from Bornholm Deep, 2 samples from Gdańsk Deep and 3 samples from Gotland Deep out of total samples of 130, were the concentrations slightly higher than values reported by Uścinowicz (2011).

The highest observed difference between given geochemical background and obtained results was observed for zinc (Table 2). Mean zinc concentrations of 193 $\mu g \cdot g^{-1}$, 180 $\mu g \cdot g^{-1}$ and 161 $\mu g \cdot g^{-1}$were reported in sediments from Bornholm, Gdańsk and Gotland Deeps respectively. In most samples, measured concentrations were nearly twofold higher than reported geochemical background levels. Potentially there could be a link between high zinc concentrations and munitions dumping operations as zinc was incorporated into various munition shells and casings. Uścinowicz (2011) suggests that elevated concentrations of zinc in the Baltic Sea basins are the results of the transport of contaminants from distant coasts.

3.3. Biota

Even during their undisturbed state, Baltic Sea deeps had relatively low biodiversity when compared to Baltic Sea coastal zones and similar depths in more saline seas (Snoeijs-Leijonmalm et al., 2017). However, it was the possible risk of CWA bioaccumulation in food chains that brought wide scientific interest in research of interactions between benthic organisms and sea-dumped munitions. Medvedeva et al. (2009) reported enhanced numbers of mustard gas-resistant bacteria in the bottom waters at Baltic dumpsites together with a decreasing biodiversity of bacteria near the identified objects. This indicated a probable leakage of munitions content while new research from Silva and Chock (2016) describes microbial-induced corrosion of shell casings in Hawaiian dumpsites.

Meiobenthic communities at investigated CW dumpsites in years from 2014 to 2017 show a great dominance of Nematoda (Fig. 8). Nematoda were the only noted taxon, except from the situation observed in Bornholm Deep in March 2016, when singular individuals of Polychaeta, Oligochaeta, Pripulida, Ostracoda, Harpacticoida and Cnidaria appeared in the sediment samples. As for now, meiofauna was represented only by Nematoda (Grzelak and Kotwicki, 2016), which also included first observations of ovoviviparous reproductive behaviour of *Halomonhystera disjuncta* in the Baltic Sea. In similar studies, Kotwicki et al. (2016), observed statistically significant differences between Nematoda communities from dumpsites in Bornholm, Gdańsk and Gotland Deeps that were most likely linked with differences of type, source and quantities of deposited OM.

Observation of single-year shift in local biodiversity of meiofauna, can be only explained as a temporary effect of MBI from 2014, which, as already mentioned, reached Bornholm Deep in March 2015, back when only Nematoda individuals were noted (Fig. 8). It turns out that favourable conditions lasted at least until March 2016 allowing i.e. a Copepod: Thalestridae (Fig. 9), belonging to the order Harpacticoida, characterized by relatively high oxygen demand (Giere, 2009), to colonize investigated areas. Undisputed dominance of Nematoda returned in March 2017, when all other taxa already disappeared.

March 2016 was also exceptional for macrofauna communities' status, as during the ROV inspections of Bornholm Deep, numerous active individuals of benthic Amphipoda were observed directly in the dumpsite area, however no recorded footage is available. At depths exceeding 90 m, it has probably been *Monoporeia* sp. (Snoeijs-Leijonmalm et al., 2017) and was also most likely an effect of the 2014 MBI. As for now, the total absence of benthic macrofauna, in the exact areas of CW dumpsites in the Baltic Sea was reported (Grzelak and Kotwicki, 2016).

Temporal returns of macrofauna and increases in oxygen levels are therefore likely to attract larger numbers pelagic and demersal fish to stay longer in CWA contaminated areas. Some species of demersal fish are already present, since individuals of European flounder (*Platichthys flesus*) and Atlantic cod (*Gadus morhua*) were observed both before, during and after the MBI thanks to the ROV investigation of sea-dumped

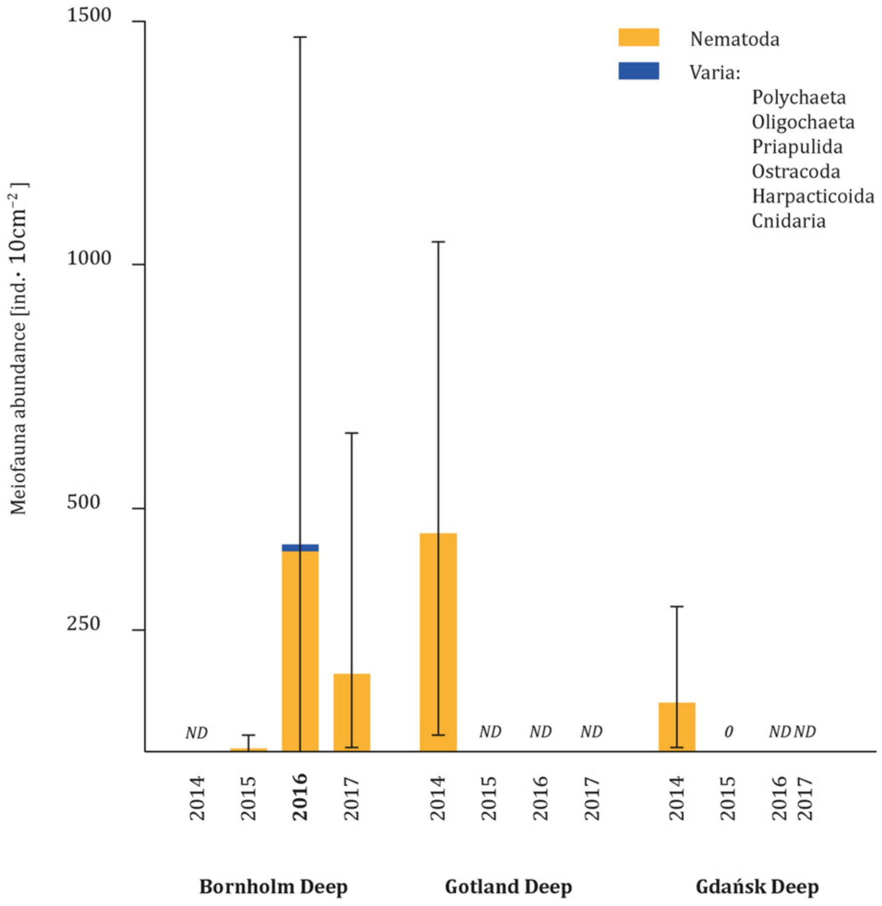

Fig. 8. Meiofauna taxa noted in sediments collected at CW dumpsites from Bornholm, Gotland and Gdańsk Deeps. Graph visualises maximum and minimum numbers of individuals found in samples and mean quantities from a site. Most dominant taxon – Nematoda, was present in all investigated samples, beside one from September in 2015 from Gdańsk Deep, when no organisms were observed. Blue colour represents other taxa: Polychaeta, Oligochaeta, Priapulida, Ostracoda, Harpacticoida and Cnidaria, that were present only in sediment samples from Bornholm Deep collected in March 2016. ND – no data.

Fig. 9. Individual of Harpacticoida belonging to Thalestridae Sars, 1905 found in one of the subsamples collected by means of VanVeen during S/Y Oceania cruise in March 2016.

munitions (Fig. 10). *G. morhua* is one of the most abundant demersal fish species inhabiting almost the entire Baltic Sea.

Two cod stocks have been identified in the Baltic Sea: the western and the eastern stock. First, primarily inhabits the Danish Straits and Arkona Deep, while the second, often considered to represent a subspecies (*Gadus morhua callarias* L.), is abundant in the Deeps of Arkona, Bornholm, Gdańsk, and Gotland up to the western Gulf of Finland. The geographical borderline between these stocks is assumed to be at the island of Bornholm (Aro, 1989).

The two Baltic cod stocks mainly differ in spawning season and the preferred spawning grounds. While the western stock spawns from February to May, the eastern stock spawns from June to September. In present times, the more successful spawning sites of the eastern stock include the deepest parts of the Bornholm Basin and the Arkona Sea (Bagge et al., 1994; Wieland et al., 2000). Spawning activity and the success of cod are greatly influenced by hydrographic conditions, including the MBI. Because of their specific density, spawned and fertilised eggs of Baltic cod require a minimum salinity of ≥ 11 to prevent them from sinking into the anoxic conditions of the bottom. For the successful survival and development of embryos, a minimal oxygen concentration of $\geq 2\ \mathrm{ml \cdot L^{-1}}$ is required (Nissling et al., 1994, Wieland et al., 2000). Since the Bornholm Basin is not only the most important spawning area for eastern cod, but also the major CWA dumpsite in the Baltic Sea, a risk of exposure to CWA for both early life stage and adult cod during their stay in the spawning grounds cannot be excluded.

According to Eero et al. (2015), the development of the Baltic cod stock is subject to numerous ecological factors that are still poorly understood. Stressors which may negatively influence the health of cod stocks, and which have so far been largely neglected in stock assessments, might be due to the effects of contaminants to which fish are exposed and/or to infection/infestation with diseases and parasites. To assess the impact of such environmental stressors, cod have been studied on a regular basis as part of national environmental monitoring programmes (Lang, 2002). Methodologies applied in fish health monitoring are mainly based on the activities of Expert Groups under the International Council for the Exploration of the Sea (ICES) (Bucke et al., 1996).

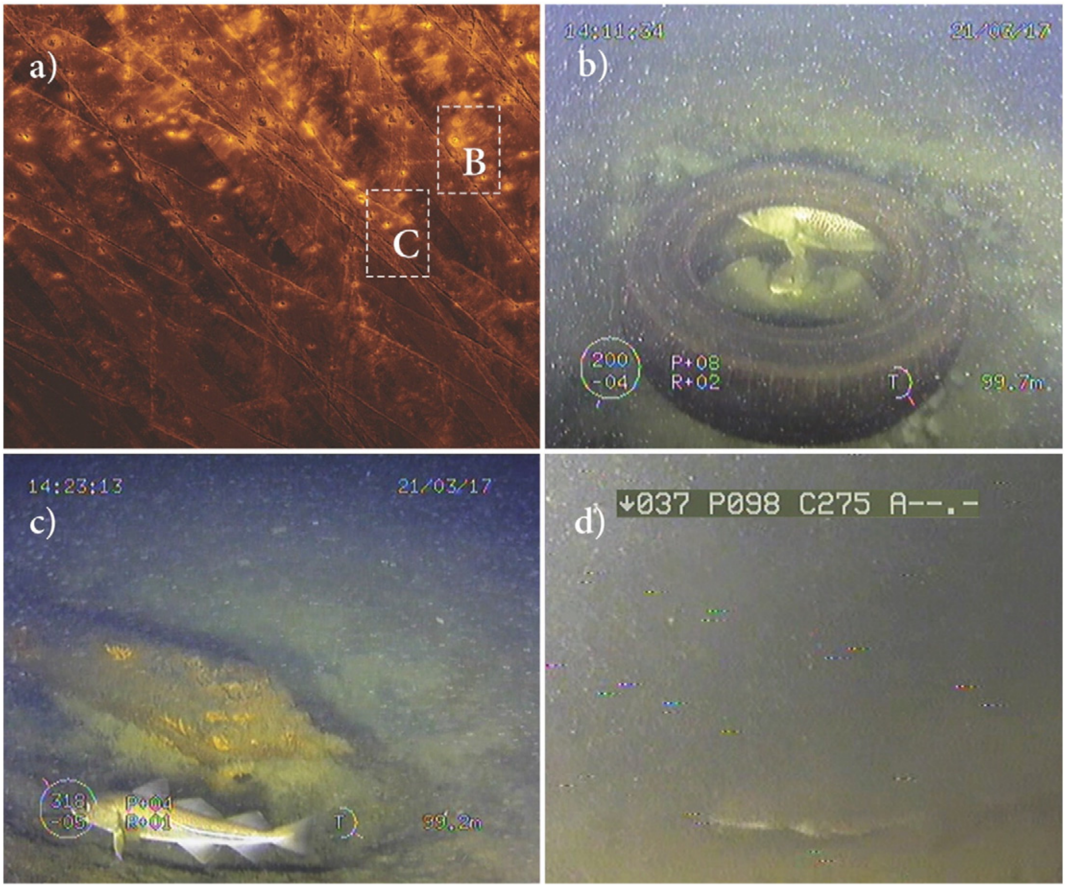

Fig. 10. Side scan sonar acoustic mosaic from March 2017 CW dumpsite area investigation of Gdańsk Deep (a), with corresponding ROV images (b - B and c - C). Atlantic cod (*Gadus morhua*) was regularly observed in close vicinity of various anthropogenic debris like tires (b) and gas tanks (c). Other specimen of demersal fish - European flounder (*Platichthys flesus*), was noted mostly in Bornholm Deep (d). Presented ROV images were taken at depths ranging from 98 to 99,7 m.

Table 3
Habitat characterization of investigated CW dumpsites.

Area	Habitat classification	CW dumpsite			Environmental conditions		Fauna		
		Status	*CWA*	*Objects*	*Dynamics*	*DO*	*Meio*	*Macro*	*G. morhua*
Bornholm Deep	Deep Sea Muddy Sands	Official	+	316	Accumulative basin	Oxic **Hypoxic** Anoxic	+	*Monoporeia* sp.	East/West
Gotland Deep	Deep Sea Muddy Sands	Official	+	76	Accumulative basin	**Hypoxic** Anoxic	+	Not observed	East
Gdańsk Deep	Deep Sea Muddy Sands	Unofficial	+	313	Accumulative basin	**Hypoxic** Anoxic	+	Not observed	East

DO – dissolved oxygen (bold – most dominant conditions); + - detected presence; East/West – spawning area for Eastern or Western stocks of Baltic Cod (*Gadus morhua*).

Bełdowski et al. (2016a) applied these methodologies along with the measurement of other biological indicators to study adverse effects of dumped CWA on the health status of cod. The results revealed no major difference in prevalence of common, externally visible diseases and parasites between cod from the deep dumpsites in the Bornholm and Gotland Basins and the reference sites. However, cod from the Bornholm dumpsite were characterized by low condition indices and some stress responses (kidney pathology, biomarkers for genotoxicity, neurotoxicity and oxidative stress) compared to cod from the other areas studied.

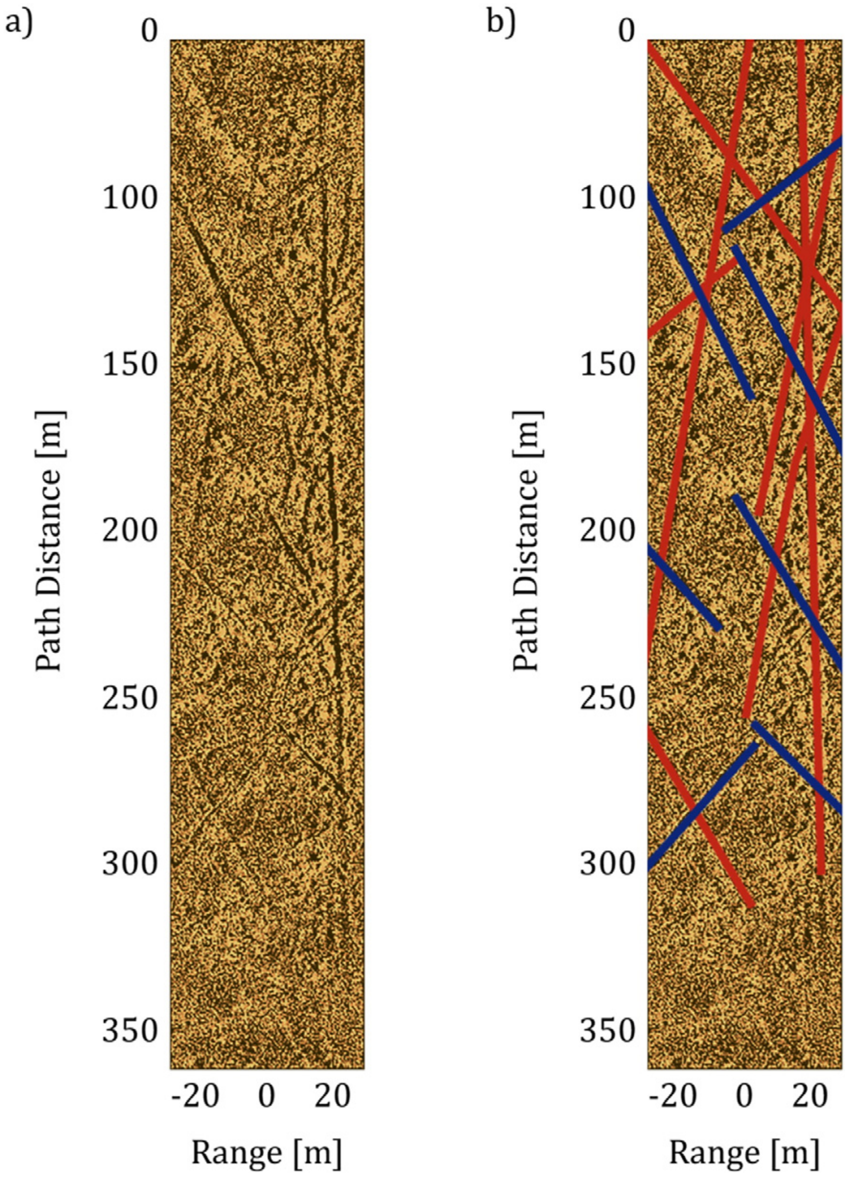

Fig. 11. Examples of high resolution (900 kHz frequency) side-scan sonar images of sea bottom with visible trawl marks. Post processing was performed for better visualisation. Colour-marked lines present supposed generations of scars (b) – younger as blue and older as red. The images were obtained near the Bornholm dumpsite at 55°19.2′N, 15°37.6′E.

3.4. Anthropogenic disturbances

In overall, 705 suspicious objects lying on top and beneath the sediment surface, including actual wrecks, bombs, mines, torpedoes and barrels, have been recorded by acoustic and magnetic methods during the investigations of the three dumpsites (Table 3). 90% of those objects were detected in Bornholm and Gdańsk Deeps.

Despite the prohibition of fishery activities in CW dumpsites, traces of bottom trawling are commonly visible on the sonar images in all investigated areas (Fig. 11). In general, the trawl marks are linear or faintly curvilinear. The images retrieved from the AUV sonar show many generations of scars. Apart from differences in sharpness of plough edges and their depth, the long history of trawling is especially visible in points where lines intersect. Beyond their influence on faunal assemblages, trawling is likely responsible for the dispersion of munitions into nearby areas.

4. Conclusions

Detected presence of sea-dumped munitions both on top and beneath sediments in all three studied areas confirms their CW dumpsite status. According to performed studies, all investigated areas fall within habitats described by EUNIS as A6.4 - Deep Sea Muddy Sands (Hill et al., 2004). There is also a complexity of factors governing ecological interactions in the CW dumpsites located in deep parts of the Baltic Sea, however, the limitations of dissolved oxygen concentrations play the most crucial role in shaping the quality of investigated ecosystem. Observed differences in various parameters among the sites are results of their varying geographical locations, bathymetry, local hydrological regimes and the sources and types of suspended material. It is worth noting that the conditions at each dumpsite correspond with the overall situation of the whole Baltic Proper. Although arsenic concentrations can still be recognized as a possible indicator of CWA and their degradation products presence in Baltic Sea sediments, the observed variability of background concentrations makes the actual anthropological sources hard to spot.

The biodiversity of all studied areas is low, however, due at to recent hydrological phenomena, the 2014 MBI and the replenished oxygen supply at depth, we have observed significant changes in both meiofauna and macrofauna assemblages in the dumpsite area of the Bornholm Deep. Regardless of the MBI, two species of demersal fish were observed in three investigated dumpsite areas during the whole lifespan of CHEMSEA and MODUM projects. On the other hand, temporary improvements of oxygen conditions in bottom waters are believed to increase the risk of fish exposure to possible negative effects originating from CWA presence in the sediments. Due to numerous environmental disturbances that already exist in the Baltic Sea, in situ verification if there is a significant threat caused by sea dumped CW for biota is difficult. Presented results should serve as a baseline comparison data for further studies to determine what are the actual effects of sea-dumped CW on Baltic Sea ecosystem.

Acknowledgments

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Acute aquatic toxicity of sulfur mustard and its degradation products to Daphnia magna.

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Acute aquatic toxicity of sulfur mustard and its degradation products to *Daphnia magna*

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ABSTRACT

Sulphur mustard (HD) was the most widely produced chemical warfare agent (CWA) in the history of chemical warfare (CW). Simultaneously, the loads of HD account as by far the largest fraction of the sea-dumped CW. Nowadays its presence in the marine ecosystems recognized as a serious threat for marine users and maritime industries. Although, during over a decade of research much has been done to assess the environmental threats linked with underwater chemical munitions. There are, however, essential gaps in scientific knowledge including scarce information about the aquatic toxicity thresholds of HD and its degradation products.

Standardized biotests were performed according to the Organisation for Economic Co-operation and Development (OECD) Test No. 202: *Daphnia sp.* Acute Immobilisation Test guidelines. Obtained results provide a solid foundation for comparison and categorisation of threats of HD and its degradation products. With the *D. magna* LC_{50} aquatic acute toxicity threshold at as low as $224 \pm 12\ \mu g \times L^{-1}$, 1,2,5-trithiepane is very toxic, being one of the most toxic CWA degradation products that have been investigated up to date. It exhibits stronger effects than 1,4,5-oxadithiepane and diluted HD that turn out to be toxic. In total, the toxicity of 7 compounds has been estimated. Whenever possible, toxicity thresholds were compared with previously existing data originating from different biotests and mathematical modelling.

1. Introduction

Sulfur mustard (Yperite, H, HD) is an infamous Chemical Warfare Agent (CWA) that had been weaponized since 1913 and been widely used on the World War I battlefields since 1916. This colourless liquid, due to the low values of vapour pressure and melting point, could only be deployed by means of grenades, artillery shells, aerial bombs or spraying, it became known as the "King of the Battle Gases" (Konopski, 2009). It is estimated that 10 811 to 12 400 tons of sulfur mustard had been produced between 1913 and 1918, with only 1300 tons remaining in post WWI arsenals (Makles and Śliwakowski, 1997; Smart, 1997; Coleman, 2005 and Konopski, 2009). Nowadays, those post-war HD residues in Western Europe have been detected in groundwater at areas of high exposure decades after its use (Chmielińska et al., 2019). Moreover, in order to improve the physical properties of sulfur mustard, it was produced in mixtures e.g. with arsine oil by Germany or with Lewisite by the United States and Russia (Söderström et al., 2017), that are now recognized to be toxic in the aquatic conditions (Czub personal communication). Although since the 1925 the Geneva Protocol prohibited the use of Chemical Warfare (CW) in battlefields, HD was produced in extensive numbers until the end of the World War II by the majority of the armies involved in the conflict.

In opposition to the battlefields of WWI, the use of poisonous substances during WWII in Europe was scarce and accidental (Konopski, 2009), however, the Japanese Army extensively used CW against China (Brombach, 2011). While the total global production of CWAs until 1945 is estimated to be around 500 000 tons, the reported production of sulfur mustard is between 206 000 to 257 000 tons (SIPRI, 1971), however the

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actual numbers are difficult to assess. At the end of the WW2 at least 28 000 tons of HD were stockpiled in German warehouses, 41 000 tons in Great Britain, from 28 000–77 000 tons in USSR, 8000 tons in Japan (mixed with Lewisite and phosgene) and 91 100 tons in USA that served as a warfare agent in around 1 000 000 bombs (Makles and Śliwakowski, 1997; Smart, 1997; Coleman, 2005, Konopski, 2009). Such CW stocks, being problematic in safe storage, required a worldwide rapid utilization (Makles and Tyszkiewicz, 1992). Sea-dumping became one of the most common method of their disposal (Long, 2009; DEPA, 2010; Edwards and Bełdowski, 2016). In total, based on multiple reports (i.e. Bearden, 2001; Missiaen and Henriet, 2002; Carton and Jagusiewicz, 2009; Bełdowski et al., 2016), we estimate that between 1919 and 1980, at least 76 000 tons of HD were submerged in the coastal areas of Europe, Africa, Australia, Asia and America. The Baltic Sea area of Bornholm Deep is the best described underwater CW dumpsite (i.e. HELCOM, 1994; Szarejko and Namieśnik, 2009; Knobloch et al., 2013; CHEMSEA, 2014; Czub et al., 2018). Between 40 000 to 65 000 tons of chemical weapons containing from 11 000 to 15 000 tons of CWAs were dumped in the Primary and secondary dumpsites (HELCOM, 1994; Knobloch et al., 2013). In total, up to 80% of those munitions had been filled with sulfur mustard as the main warfare agent (Knobloch et al., 2013).

According to Munro et al. (1999), in typical conditions, sulfur

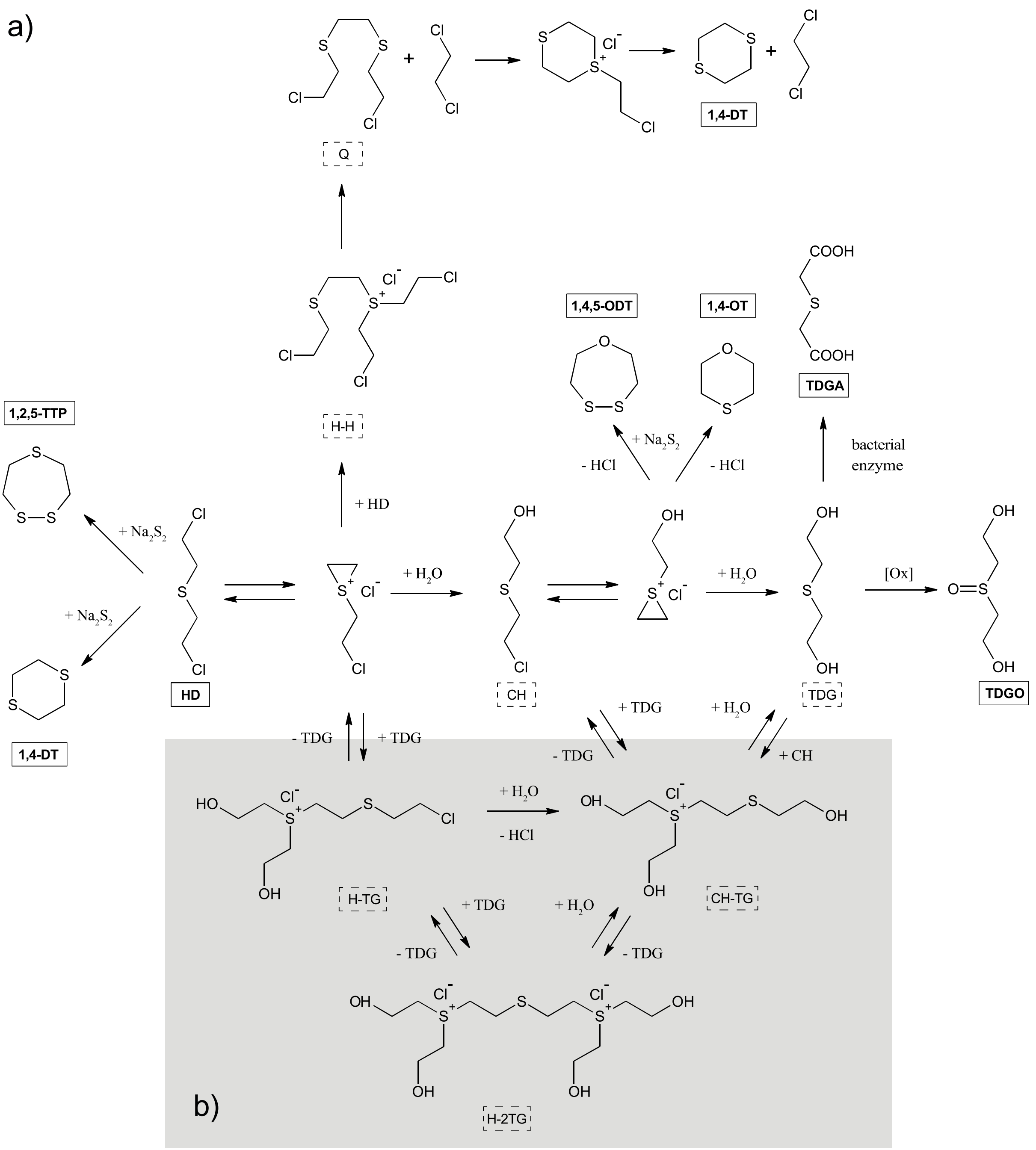


Fig. 1. Sulfur mustard (HD) hydrolyzation and transformation in the aquatic conditions into a) thiodiglycol sufloxide (TDGO) thiodyglicolic acid (TDGA) and cyclic degradation products: 1,4-Dithiane (1,4-DT); 1,4-Oxathiane (1,4-OT); 1,4,5-Oxadithiepane (1,4,5-ODT); 1,2,5-Trithiepane (1,2,5-TTP) that were tested in this study, TDG – thiodiglycol; CH – mustard chlorohydrin; Q – Sesquimustard and H–H – product of HD-HD condensation that were not tested in this study and b) water-soluble degradation-polymerisation products (not tested in this study): H-TG – product of HD-TDG condensation; CH-TG – product of CH-TDG condensation and H-2TG – product of TDG-H-TG.

mustard is expected to form – through a rapid hydrolysis – thiodiglycol (TDG) which can later undergo oxidation into thiodiglycol sulfoxide (TDGO). However, especially in the Baltic Sea dumpsites, HD due to its low water solubility, viscosity and hydrophobic properties often encapsulates itself in a form of persistent lumps (Söderström et al., 2017). Their presence in the sea bottom poses a serious risk for the fishermen and offshore workers (Makles and Tyszkiewicz, 1992; CHEMSEA, 2014). On the other hand, the ecological threat from HD was often negated (Andrulewicz, 1996). According to Sigma-Aldrich safety data sheets that can be found on the Organization for the Prohibition of Chemical Weapons (OPCW) website (www.opcw.org), TGD is known to be non-toxic, with *D. magna* acute toxicity threshold above 500 mg × L^{-1}. In 1998-99, detailed laboratory tests of the sulfur mustard lump caught on 9 January 1997 revealed presence of 50 various chemical compounds, while their chemical structure was identified in 30 cases. Those included sesquimustard (Q), its analogues as well as oxygenic compounds. However, back then, no TDG was detected, probably due to high water-solubility of this compound (Mazurek et al., 2001). More recent analyses of the sediment contamination in the Baltic Sea dumpsites (Nawała et al., 2016) report the detections of HD at its intact form (in several sediment samples), together with a wider range of degradation products. Both TDG and TDGO are rarely found in sediment samples from the marine CW dumpsite areas. Instead, mustard heel-related compounds are observed (Fig. 1). Sulfur mustard heel consists of water-soluble degradation-polymerisation products (Fig. 1b) and cyclic degradation products (Fig. 1a), e.g. 1,4-dithiane and 1,4-oxathiane, which can undergo further transformations (Rohrbaugh and Yang, 1997; Wagner et al., 1999; Söderström et al., 2017). In the Hawaiian site investigated by the HUMMA project mustard degradation products were detected as a thin film covering the sea bottom nearby munitions, that tended not to mix with the sediment. This was probably caused by the depth of the site (ca. 600m), which is associated with low current speed, and possibly other composition of mustard in terms of added thickeners (Edwards et al., 2016). HD was detected in only one sample, while remaining samples contained 1,4-dithiane and 1,4-oxathiane (Briggs et al., 2016). Contrary to the Hawaiian dumpsites, in the Baltic Sea, a film coating at the sediments was not present, but the CWA degradation products were incorporated into bulk sediments (Bełdowski personal communication). This should be linked with the hypoxic and anoxic conditions in the Bornholm Deep dumpsite at depths around 90 m (Czub et al., 2018), as the main pathway of sulfur mustard degradation is by hydrolysis followed by the transformations with abundant ambient sulfides (Vanninen et al., 2020). Furthermore, when dispersed in sediments, sulfur mustard may undergo additional transformation by biotic processes (e.g. bacteria-mediated biotransformation). This was substantiated by the discovery of mustard resistant bacteria in the dumpsite (Medvedeva et al., 2009). Formation of degradation products that are more water-soluble and more persistent than the parent compound, increase the potential spreading and bioavailability, hence the overall potential environmental impact of HD-related threats.

Recent findings indicate that sea-dumped CWAs may potentially bioaccumulate or induce negative effects on the aquatic biota at all levels (Kotwicki et al., 2016; Lang et al., 2017; Nawała et al., 2016; Chmielińska et al., 2019). Contact with sulfur mustard, which is a vesicant compound, cyto- and genotoxic, is known to be extremely dangerous for humans, terrestrial mammals vertebrates and fish (i.e. Smart, 1997; Amato et al., 2006 and Lang et al., 2017). Munro et al. (1999) report toxicity thresholds for HD inhalation in various terrestrial species. In this context it is surprising that Muribi (1997) and Missiaen and Henriet (2002) reported lack of its effects on *Daphnia magna* exposed to concentrations below 0.5–1.0 mg × L^{-1}. A different mustard compound, a nitrogen mustard (HN2), is on the other hand known to be toxic for aquatic species (Lan et al., 2005). Although much has been done to understand the potential environmental impact of sea dumped CWAs (Sanderson et al 2007, 2010; Czub et al., 2018), including HD and its degradation products, there is still a significant knowledge gap regarding the toxicity of those compounds in the aquatic conditions. The main objective of this work was to establish the aquatic acute toxicity thresholds, according to Globally Harmonized System of Classification and Labelling of Chemicals (UN, 2019), for HD and six HD-related compounds that are found in the CW-dumpsite sediment samples.

2. Methods

2.1. Test design

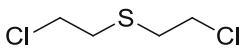
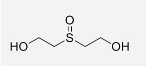
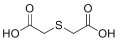
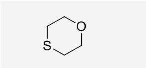
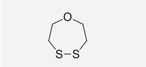
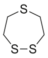
The OECD Test No. 202: "*Daphnia sp.* Acute Immobilisation Test" (OECD, 2012) was applied to test the toxicity effects of 7 substances that have been detected in sediment samples from the Baltic Sea CWA dumpsites (Table 1). The list includes one OPCW A-listed CWA: sulfur mustard (HD) and its degradation products: thiodiglycol sulfoxide (TDGO), thiodiglycolic acid (TDGA), 1,4-dithiane, 1,4-oxathiane, 1,4,5-oxadithiepane and 1,2,5-trithiepane. All tests were performed using either the third or fourth clutch of *D. magna* individuals (aged less then 24 h) of a clone (DMB) originating from Grosser Binnensee (Germany), a coastal lake with temporal intrusions of saltwater from the Baltic Sea, thus it can be considered as representative for both freshwater and brackish biota. The *D. magna* clone was maintained at facilities of the Department of Hydrobiology at University of Warsaw. Depending on the number of hatched organisms at each synchronized cohort, a range from five to nine tested concentrations per substance was arranged to fit a geometric series with a separation factor of 2.0, with an exception for one HD toxicity threshold testing with a separation factor of 1.4. Batch-culture design of experiment was applied. The experiments were conducted in beakers filled with 0.1 L of medium. Each treatment consisted of four replicates (beakers). Eight unfed animals were kept in each replicate for 48 h. Borosilicate glassware was used to minimize adhesion of the tested substances. After spatial randomization the beakers were covered using a Whatmann (UK) filtering paper to prevent contamination by dust.

To comply with the test validation criteria, all biotests were run in a water bath at a constant temperature of 21.0 ± 0.5 °C. The tests were performed under 16:8 L:D cycle. Light intensity was measured using a Li-Cor Biosciences® (USA), 189 quantum sensor measuring radiance and was constant during all the performed tests (0.27–0.36 μmol × m^{-2} × s^{-1}). Dissolved oxygen concentrations were measured using an YSI ProODO® (USA) hand-held dissolved oxygen meter, while pH was measured using Mettler Toledo® (Switzerland) F20-Standard FiveEasy Benchtop F20 pH/mV Standard Kit with LE438 sensor. Both variables were measured in all control beakers and in all the highest concentrations at the beginning and at the end of each experiment.

2.2. Preparation of solutions

1,4-oxathiane 99%, 1,4-dithiane, 98.5%, TDG 99% and TDGA 99% were purchased from Sigma Aldrich (St. Louis, USA). Sulfur mustard, TDGO, 1,4,5-oxadithiepane and 1,2,5-trithiepane were synthesized at Military University of Technology in Warsaw in small amounts, with the purity at least 98%. Purity of the compounds was measured by LC-MS and GC-MS. Apart from sulfur mustard, the stock solutions were prepared by dissolving known amounts of tested compounds in medium (using sonification to ensure that dissolving was complete) and were later filtered through Whatmann (UK) GF/C fiberglass filters and aerated for at least 48 h before use. The medium was the same conditioned lake water that is being used for *D. magna* stock cultivation and reproduction in facilities of the Department of Hydrobiology at University of Warsaw. Medium was filtered through Whatmann (UK) GF/C fibreglass filters and aerated for at least 48 h before its use. Because of low solubility of HD in water, it had been dissolved in acetone from Sigma Aldrich (St. Louis, USA) prior to the preparation of experimental media. Constant volume of acetonic solution of HD was used to prepare medium for each HD concentration (100 µL per 0.1 L of water) and for

Table 1
List of tested compounds, including their detections by CHEMSEA, MODUM and DAIMON projects (CHEMSEA, 2014; Nawała et al., 2016; Magnusson et al., 2016; Christensen et al., 2016; Chmielińska et al., 2019). It is worth underlining that there are significant differences in 1,4-dithiane solubility provided by a) Munro et al., (1999), b) Chmielińska et al., (2019) and c) maximum experimental data determined by GC-MS/MS analyses during this study.

	NATO	CAS#	Formula	Type/Source	Physico-chemical parameters		Highest detection in the Baltic Sea	
					Solubility in water	*Vapour Pressure* [Pa]	*Sediments dry weight* [μg × kg^{-1}]	*Pore Water* [μg × L^{-1}]
Sulfur mustard	HD	505-60-2		CWA	0.608 g × L^{-1}	9.33	203.7	Not Detected
Thiodiglycol sulfoxide	TDGO	3085-45-8		Degradation product of **HD**	1000 g × L^{-1}		603.9	Not Detected
Thiodiglycolic acid	TDGA	123-93-3		Degradation product of **HD**	400 g × L^{-1}		551.4	Not Detected
1,4 - Oxathiane	1,4-OT	15 980-15-1		Degradation product of **HD**	65.627 g × L^{-1}	614	252.2	Not Detected
1,4 - Dithiane	1,4-DT	505-29-3		Impurity of **HD** (production) Degradation product of **HD**	3.0 g × L^{-1} [a] 22 040 g × L^{-1} [b] 0.028 g × L^{-1} [c]	6.2	999.8	3.4
1,4,5 - Oxadithiepane	1,4,5-ODT	3886-40-6		Degradation product of **HD**	4.667 g × L^{-1}	47	2585.8	19.0
1,2,5 - Thritiepane	1,2,5-TTP	6576-93-8		Degradation product of **HD**	0.700 g × L^{-1}	8.6	2216.8	0.76

solvent control treatment (100 μL of acetone free of HD per 0.1 L of water). The resulting acetone concentration in experimental media (78.45 mg × L^{-1}) were below the 48h LC50 threshold concentration of 9218 mg × L^{-1} and NOEC at 403 mg × L^{-1} according to Cowgill and Milazzo (1991).

In order to validate the overall data acquisition and to account for possible time effects each of the tested CWAs was tested twice, which provided two independent response curves for each substance. Thus, a total of 14 tests were performed under the controlled conditions.

In one case, when testing HD toxicity, in addition to standard OECD Test No. 202 setup, we established an additional treatment that consisted of four repetitions with 8 *D. magna* individuals in each, which were exposed to the presence of the smallest volume of pure, undiluted HD, that was forming a single droplet (40 μL) at the bottom of the 0.1 L glass beaker.

Basic chemical and physical parameters of water used for the preparation of experimental media (pH, conductivity, temperature and salinity) were monitored using YSI EXO1 (USA) probe. Additional analysis of the total organic carbon (TOC) of the medium were performed using Multi N/C® 3100 analyser from Analytik Jena AG (Germany).

2.3. *Chemical analyses*

Several series of gas chromatography-tandem mass spectrometry (GC-MS/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed to provide real-data concentrations for the most accurate estimation of the toxicity thresholds (Nawała et al., 2016). Analysis were performed in two ways: intact analyses by GC-MS/MS and intact analyses by LC-MS/MS. The SRM (selected ion monitoring) transition were shown at Table 2. Analytical values of all tested concentrations of were determined by either GC-MS/MS or

Table 2
Validation of selected parameters of GC-MS/MS and LC-MS/MS methods used for analysis of tested chemicals.

	Analysis method	Parent ion [*m/z*]	Product ion	Collision cell energy [eV]	LOD [ng/mL]	LOQ [ng/mL]	Recovery [%]
Sulfur mustard	GC-MS/MS	109	63	5	0.02	0.06	93
		109	73	11			
		158	109	6			
Thiodiglycol sulfoxide	LC-MS/MS	139	77	8	0.3	0.08	100
		139	63	12			
		139	58	28			
Thiodiglycolic acid	LC-MS/MS	149	105	5	0.06	0.19	100
		149	61	10			
		105	61	10			
1,4 - Oxathiane	GC-MS/MS	104	61	9	0.02	0.06	95
		104	74	7			
		104	46	11			
1,4 - Dithiane	GC-MS/MS	120	61	15	0.32	0.95	89
		120	105	8			
		92	46	11			
1,4,5 - Oxadithiepane	GC-MS/MS	136	64	9	0.24	0.73	94
		136	92	7			
		136	43	11			
1,2,5 - Trithiepane	GC-MS/MS	152	87	17	0.12	0.38	96
		152	92	5			
		152	60	11			
Thiodiglycol*	LC-MS/MS	105	45	19	0.43	1.31	100
		105	81	4			
		105	75	8			

LC-MS/MS at the beginning and at the end of all experiments in order to determine time related variations at each concentration. All measurements were repeated at least five times and the results were standardized according to Q-Dixon test.

GC-MS/MS analyses were performed with Agilent Technologies 7890A gas chromatograph coupled with Agilent Technologies 7000 GC-MS Triple Quad tandem mass spectrometer (MS/MS). The mass spectrometer was operated in the electron impact (EI) mode. The quantitative analyses of analytes by GC-MS/MS were performed using multiple reaction monitoring (MRM) mode. The analysis was completed using a BP-5 capillary column (30 m × 0.25 mm × 0.25 μm) from SGE (Mulgrave, Australia), and was carried out using the following temperature program: the column was heated from 70 to 250 °C at a rate of 20 °C × min^{-1} and the final temperature was maintained for 1 min. Helium was used as the carrier gas and the gas flow rate was 1 mL × min^{-1}. The injector temperature was around 250 °C. The temperatures of the transfer line, ion source and quadrupoles were 250, 230 and 150 °C, respectively. During the analysis, the injector was in the splitless mode and the assigned solvent delay was 2.5 min. The Agilent Mass Hunter Workstation for series triple Quadrupole vers. B06.00 build 6.0.6.25.4sp4 software was used to obtain chromatograms and to collect quantitative data. Agilent Mass Hunter Quantitative Analysis vers. B 07.00 build 7.0.457.0 software was used for chromatograms processing. For GC analysis of 1,4-oxathiane, 1,4-dithiane, 1,4,5-oxadithiepane and 1,2,5-trithiepane the liquid-liquid extraction was performed. Samples (2 mL) were transferred into a falcon tube where 2 mL of dichloromethane (DCM) were added. After 30 min of shaking at 2000 rpm, the organic layer was transferred to a different falcon tube and was dried with anhydrous magnesium sulfate ($MgSO_4$). In the following step, the samples were analysed by GC-MS/MS. The recoveries of the compounds were between 75 and 90%.

LC-MS/MS analysis were performed with the Agilent Technologies 1260 Infinity liquid chromatograph equipped with a tandem mass spectrometer Agilent Technologies 6460 Triple Quad LC/MS using the MRM mode. Reversed-phase gradient separation was achieved on an SB-C18 column (2.1 × 100 mm, 1.8 μm). The mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The following multigradient steps were used: 5% B, increased to 100% after 5 min and held for 5 min, and returned to 5% B. The column was kept at 30 °C and the injection volume was 10 μL. MS detection was carried out using atmospheric pressure chemical ionization (APCI) in the positive ion mode. The following operating conditions were used: drying gas temperature 350 °C, vaporiser temperature 400 °C, drying gas flow 5 mL × min^{-1}, corona current 5 μA, capillary voltage 3.5 kV, nebulizer pressure 35 psi and fragmentor voltage 80V. Data were collected and handled via Muss Hunter B.07.00 (Agilent Technologies). TDG, TDGO and TDGA samples were filtered through the syringe filter with 0.15 μm pore size and no additional sample preparation was required. Due to the rapid hydrolysis of sulfur mustard in experiment condition the amounts of HD were determined by measuring the TDG concentration in the samples. In order to achieve the complete hydrolysis of sulfur mustard before analysis the samples were kept at 40 °C for 60 min.

Additionally, the sulfur mustard hydrolysis kinetic curve was obtained using GC-MS/MS and liquid-liquid extraction. At the beginning the determined amount of HD was added to water and then the measurement of time started. Then at appropriate time intervals the 2 ml of samples were transferred into a falcon tube containing 2 mL of dichloromethane DCM and 0.1g of NaCl. After 30 min of shaking at 2000 rpm, the organic layer was transferred to a different falcon tube and was dried with anhydrous magnesium sulfate ($MgSO_4$).

2.4. LC_X, EC_X and NOEC estimation

Immobilization and mortality of *D. magna* were recorded after 24 and 48 h by visual inspection of each beaker. Due to the rapid hydrolyzation of sulfur mustard, the effects of its exposure were additionally recorded

Table 3

Results from 14 performed OECD 202: *Daphnia magna* Immobilization Tests assessing acute toxicity of sulfur mustard and its degradation products at MUT laboratories. Numbers in *Conc* column represent the amount of tested concentrations during each test; *Indv./Tot.* - number of organisms per repetition and the total number of *D. magna* individuals used in the test, while Roman letters in the upper index represent the generation of the cohort. **O_2 –** mean Oxygen concentration and mean pH measurement in vessels with a highest tested concentration (A) at the beginning (0h) and at the end (48h) of the experiment. **Concentration (A)** - values of the highest analysed concentration (*0h* - initial *48h* – final); *Δ* represents time-related difference; *Error* indicates the standard error of performed chemical analyses. Due to the rapid hydrolysis, the concentrations of HD were determined using TDG as proxy. Bolded CWA LC_{50} and EC_{50} values are the most trustworthy with either effects in lower concentrations or concentration-response curve characterized by stronger statistics^a – $p < 0.001$ while^b – $p > 0.1$. **NOEC** is a highest concentration at which no observable effects were reported. The acute aquatic toxicities of thiodiglycol (TDG) and nitrogen mustard (HN2) are after [1]- Sigma-Aldrich safety data sheets that can be found on the Organization for the Prohibition of Chemical Weapons (OPCW) website (www.opcw.org) and [2] – Lan et al. (2005). Toxicity thresholds are applied according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

		OECD 202: *Daphnia magna* Immobilization Test														
		Date	*Conc*	*Indv./Tot.*	O_2 [mg × L^{-1}]		pH		Concentration (A) [μg × L^{-1}]				Toxicity thresholds [μg × L^{-1}]			
					0h	*48h*	*0h*	*48h*	*0h*	*48h*	*Δ*	*Error*	LC_{50}	EC_{50}	NOEC	
Sulfur Mustard	**HD**	*05.06.19*	**9**	$8/320^{IV}$	8.9	8.7	8.2	8.2	99 799.5	96 996.2	97.2%	4.4%	**9670 ± 621** ^{a}	**8916 ± 518** ^{a}	3627 ± 279	**Toxic**
		26.08.19	**9**	$8/320^{III}$									13 653 ± 2958^a	12 352 ± 386^a		
Thiodiglycol sulfoxide	**TDGO**	*05.06.19*	**5**	$8/192^{IV}$	9.3	9.1	8.4	8.1	98 392	96 662.2	98.2%	2.8%			98 392 ± 2767	Non-toxic
		11.06.19	**5**	$8/192^{III}$												
Thiodyglicolic acid	**TDGA**	*22.05.19*	**5**	$8/192^{III}$	9.1	8.9	7.8	7.7	106 657.1	103 884.2	97.4%	8.1%			106 657 ± 8660	Non-toxic
		31.05.19	**5**	$8/192^{V}$												
1,4 - Oxathiane	**1,4-OT**	*22.05.19*	**9**	$8/320^{III}$	9,1	8.8	8.4	8.1	108 762.3	105 177.8	96.7%	4.4%			108 762 ± 4765	Non-toxic
		29.05.19	**5**	$8/192^{V}$												
1,4 - Dithiane	**1,4-DT**	*22.05.19*	**8**	$8/288^{III}$	8.8	8.5	8.5	8.2	28 745.7	28 382.1	98.7%	8.1%			28 745 ± 2328	Non-toxic
		29.05.19	**5**	$8/192^{V}$												
1,4,5 - Oxadithiepane	**1,4,5-ODT**	*31.05.19*	**6**	$8/224^{III}$	9.1	8.7	8.1	8.0	71 727	68 607.1	95.6%	4,6%	2188 ± 154^a	2188 ± 154^a	741.97 ± 25.87	**Toxic**
		11.06.19	**5**	$8/192^{III}$									**2340 ± 159** ^{a}	**2175 ± 155** ^{a}		
1,2,5 - Trithiepane	**1,2,5-TTP**	*16.07.19*	**9**	$8/320^{III}$	8.9	8.6	8.0	7.9	836,4	834.8	99.8%	4.6%	283.83 ± 19.35 ^{b}	197.75 ± 65.90 ^{b}	37.80 ± 25.87	**Very toxic**
		19.07.19	**6**	$8/224^{III}$									**224.15 ± 11.98** ^{a}	**214.83 ± 10.41** ^{a}		
Thiodiglycol	**TDG**														>500 000 [1]	Non-toxic
Nitrogen Mustard	**HN2**														2520 [2]	**Toxic**

after 30 s, 15, 30 and 60 min. Distinction between dead and immobilized animals was on the basis of behaviour (moving of filtering appendages) and post-mortem features of *D. magna* (e.g. whitening, unnatural body position). LC_{50} values were estimated based on the observed mortality while EC_{50} values were estimated based on a summarized observed mortality and immobilization among organisms per tested concentration. Toxicity thresholds estimations were based on results from all the successfully performed biotests. Analytical values of initial concentrations of the test compounds in the test media were selected for further calculations. Concentration-response curves were created using the *drc* and *ggplot2* packages in the R Studios software (Ritz et al., 2015). Model was fitted to the Log-logistic (ED_{50} as parameter) function (LL.2, type binomial) with lower limit at 0 and upper limit at 1 (2 parameters). LC_{50} and EC_{50} values and their significance levels were calculated by the software.

3. Results

The values of nominal and analytical concentrations correspond to each other and more or less fit the geometric series factor of 2.0, as the errors of all spectrometry-based measurements for each chemical ranged between 2.8 and 8.1% (Table 3). A total of 14 tests were performed under the controlled conditions. Dissolved oxygen concentrations never dropped below 8 mg $\times$ L^{-1} either at the beginning or at the end of all the tests (Tab 3), thus, the validation criteria of the OECD test (oxygen concentration above 3 mg $\times$ L^{-1}) were met. Reported pH values varied from 7.7 to 8.5 but remained stable during the test and were corresponding to the properties and concentrations of the tested compounds. TOC values were at 1.61 $\pm$ 0.03 mg $\times$ L^{-1}.

With no observed mortality nor immobilization in any of the control nor solvent control treatments, all tests met the OECD validation criteria. Three of the tested compounds: sulfur mustard, 1,4,5-oxadithiepane and 1,2,5-trithiepane induced lethal effects on *D. magna* resulting in 100% mortality at least at the highest tested concentrations (Fig. 2). The 48h LC_{50} of 1,2,5-trithiepane was 224 $\pm$ 12 μg $\times$ L^{-1} ($p < 0.001$), and this compound turned out to be to very toxic, with toxicity thresholds $<$ 1 mg $\times$ L^{-1} (Table 3). The 48h LC_{50} of 1,2,5-oxadithiepane and sulfur mustard were higher, with 2340 $\pm$ 159 μg $\times$ L^{-1} ($p < 0.001$) and 9670 $\pm$ 621 μg $\times$ L^{-1} ($p < 0.001$) respectively. With toxic threshold below 10 mg $\times$ L^{-1} they are classified as toxic compounds. 1,4-dithiane showed no effects of exposure on *D. magna* up to its maximum solubility, while 1,4-oxathiane, thiodiglycol sulfoxide and thiodiglycolic acid

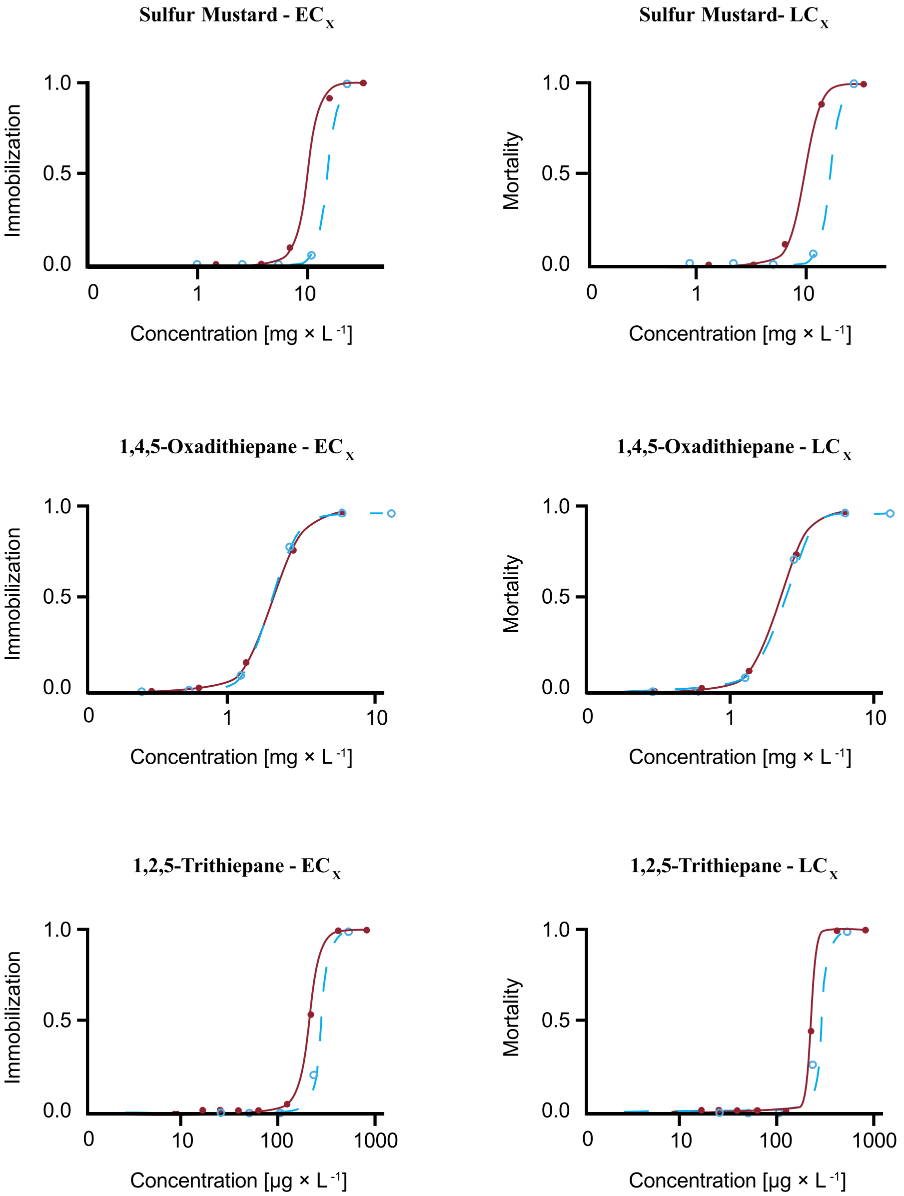


Fig. 2. Diagrammatic representation of the concentration-response curves from two test repetitions of sulfur mustard (HD), 1,4,5-Oxadithiepane and 1,2,5-Trithiepane effects on *Daphnia magna* during the 48hrs of exposure. None of the remaining HD degradation products that were tested caused any observable effects; therefore, they are not represented in this figure. Results that served as a basis for EC and LC calculations are visualized as solid lines and points, while the repetitions as dashed lines and circles.

showed no effects at concentrations of approx. 100 mg × L^{-1} (Table 3).

To estimate the duration of *D. magna* exposure to the presence of HD, its hydrolysis curves were calculated (Fig. 3). In dilution water used in this study, the half-life value of sulfur mustard at 20 °C, is at 8 min 19 s. Within 48 h, both immediate and "post-exposure" effects of HD on *D. magna* were observed (Fig. 4). At the highest concentrations (treatments A – 99.80 ± 4.57 mg × L^{-1} and B – 47.29 ± 2.81 mg × L^{-1}) first manifestation of lethal effects was observed in less than 15 min after exposure and total lethality of exposed organisms in less than 60 min. In these treatments immediate immobilization was never reversed. At medium concentrations (treatments C – 26.24 ± 0.87 mg × L^{-1} and D – 12.62 ± 0.41 mg × L^{-1}) the immediate immobilization was reversed, resulting in observations of active swimming of 22% of organisms after 30 min and 44% of organisms after 60 min from exposure. Nevertheless, it resulted in 100% immobilization after 24 h and 100% mortality after 48 h at concentration C and immobilization of 91% and mortality of 84% of organisms at concentration D after 48 h. After 48 h, the HD acute toxicity EC_{10} was recorded at concentration E (6.28 ± 0.40 mg × L^{-1}) and it was the lowest tested concentration at which any effects of exposure were recorded. NOEC for HD was at 3.63 ± 0.28 mg × L^{-1}. In addition to this, the 100% mortality was observed after 48 h in the beakers, where organisms were exposed to the presence of the raw HD in the form of a droplet (Fig. 4).

4. Discussion

Values of all measured environmental variables met the validation criteria of the test (OECD, 2012), therefore all obtained results provide a solid foundation for estimation of the acute aquatic toxicity thresholds of all investigated compounds. Our study indicates that when diluted, sulfur mustard remains toxic in water. In comparison with reported nitrogen mustard LC_{50} *D. magna* acute toxicity threshold of 2.5 mg × L^{-1}, sulfur mustard is slightly less toxic, however both compounds have LC_{50} values that fall into a "toxic" label (>1 mg × L^{-1} and <10 mg × L^{-1}). This should indicate that despite rapid hydrolyzation into the non-toxic TDG, whenever mechanically disturbed, the sulfur mustard can cause significant threat to the benthic and demersal organisms. This goes along with the observed mortality of *D. magna* in the presence of the raw HD, most likely due to a direct contact with a singular droplet. This observation stays in contrast to some older statements claiming that the HD-related environmental threats could come only from the direct contact with large sulfur mustard lumps laying on top of the sediment. Notably, breakdown of larger mustard lumps into small, pea size grains was observed in the Bornholm Deep, where fishermen sometimes observe it as a catch contamination (Sanderson, personal communication). Obtained *D. magna* HD 48 h NOEC at 3627.50 ± 0.28 mg × L^{-1} is a significant update when compared with previous studies that reported non-toxic properties of acetone-diluted sulfur mustard to *D. magna* at concentrations up to 0.5–1.0 mg × L^{-1} without estimation of the LC_{50} and EC_{50} thresholds (Muribi, 1997; Missiaen and Henriet, 2002). Every year the corrosion of munitions at the Baltic Sea bottom progresses, which will probably increase their impact on the environment in the near future (Bełdowski et al., 2017). According to corrosion models, many containers have already released toxic substances to the environment, while others could do so in next 30–40 years (Makles and Śliwakowski, 1997; Fabisiak et al., 2018). At the same time, offshore activities that could disturb munitions and accelerate this process intensify. It all results in contamination spreading and increased bioavailability, which was observed ie. based on mercury released from burster charges (Bełdowski et al., 2019).

Together with recent findings from Chmielińska et al. (2019), the confirmed toxicity of two common sulfur mustard degradation products adds a novel perspective. The *D. magna* LC50 at 224 ± 12 μg × L^{-1} makes 1,2,5-trithiepane one of the most toxic CWA degradation products that were tested so far, while according to Chmielińska et al. (2019) the 1,4,5-oxadithiepane belongs to the Chronic Category 2: "toxic to

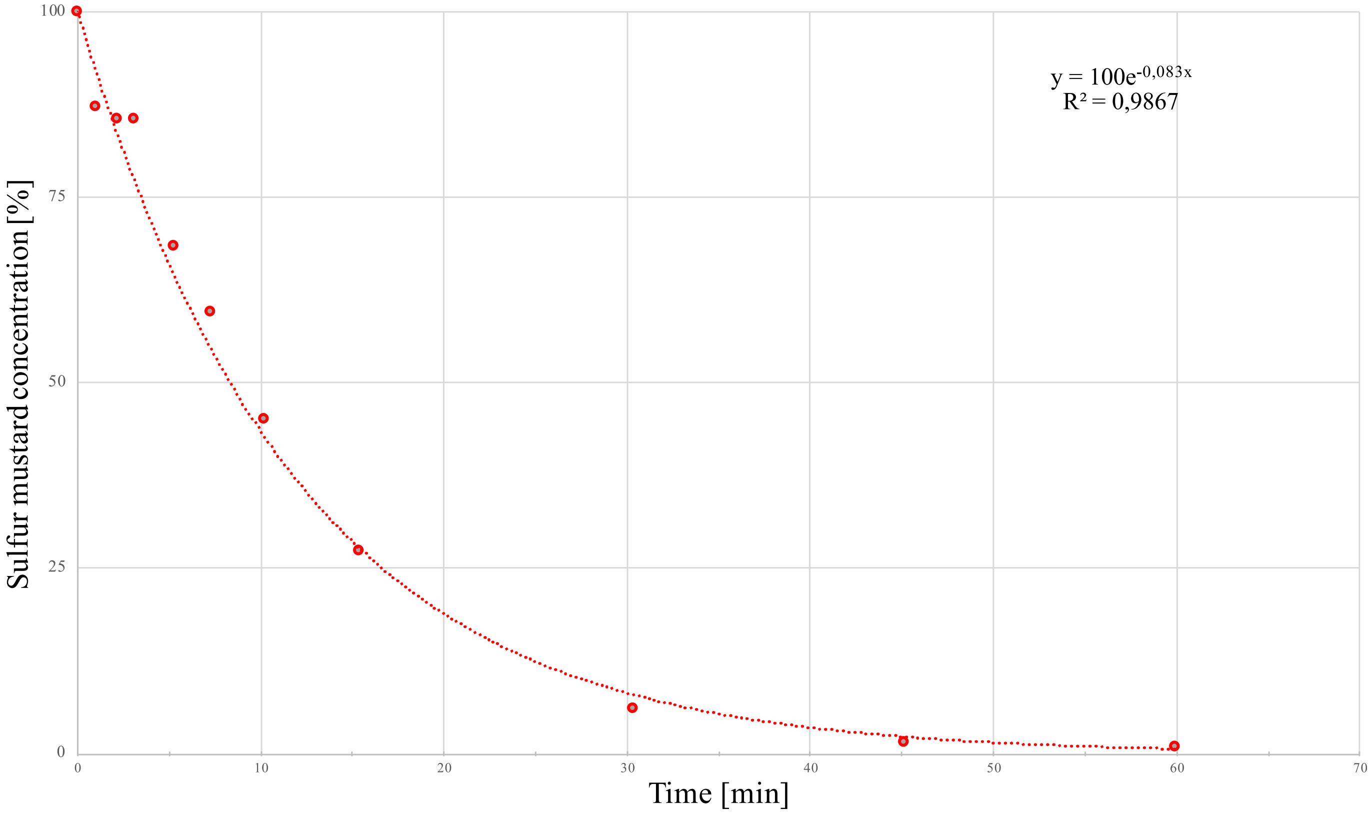


Fig. 3. Sulfur mustard (HD) hydrolysis in experiment medium at 20 °C. According to the kinetic curve, half of the initial HD concentration will hydrolyse after 8 min 19 s. At such conditions the total transformation of sulfur mustard should be expected after approximately 60 min from its initial exposure to water.

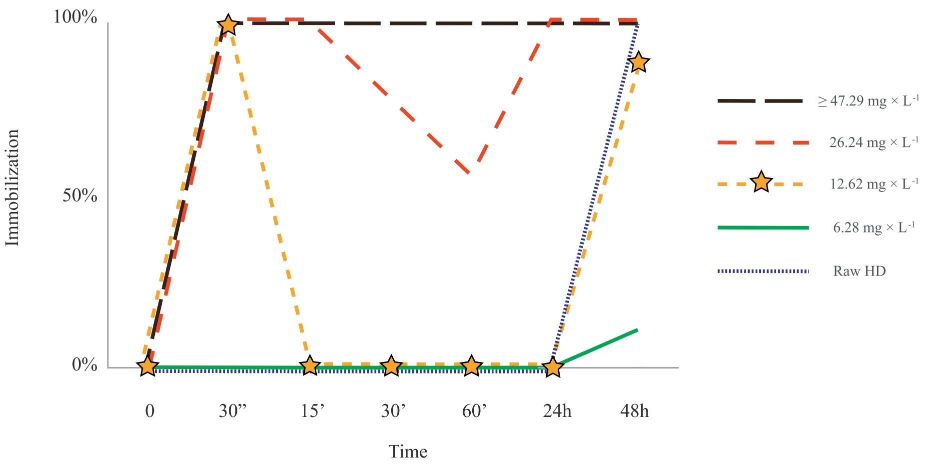


Fig. 4. Immobilization rates of *Daphnia magna* at various sulfur mustard concentrations during the 48 h exposure. Within 48 h, both immediate response and temporal recovery from the effects of HD on *D. magna* were observed The black dotted line represents the responses of daphnids exposed to two highest tested concentrations (99.80 ± 4.57 mg × L^{-1} and 47.29 ± 2.81 mg × L^{-1}). The blue dotted line represents the response of daphnids exposed to raw HD droplet (40 μL). After 48 h, the HD acute toxicity EC_{10} was recorded at 6.28 ± 0.40 mg × L^{-1}, and it was the lowest tested concentration at which any effects of exposure were recorded. NOEC for HD was at 3.63 ± 0.28 mg × L^{-1}. . . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

aquatic life with long lasting effects”. Both compounds have been detected in Baltic Sea sediments in concentrations exceeding the LC_{50} and EC_{50} values, moreover, 1,4,5-oxadithiepane was the only HD-related compound that was detected in pore-water (Table 1). The concentrations detected in pore-water at approx. 1% of the *D. magna* acute LC_{50} are also below the reported chronic (21 days) *D. magna* NOEC = 825.98 μg × L^{-1} (Chmielińska et al., 2019).

In comparison with existing data on HD and HD-related compounds toxicity (Fig. 5), results obtained in this study using *D. magna* as a model organism for the CWA risk assessment are more reliable than ECOSAR predictions. Our study also provides information about toxicity of 1,2,5-tritiepane and 1,4,5-oxaditieoane to aquatic eucaryotes that were previously non available, nevertheless this information is corresponding with Microtox™ data from Christensen et al. (2016), who reported both compounds to have negative effects on the test bacterium *Allivibrio fischeri* (Fig. 3). On the other hand, TDGO, TDGA, 1,4-oxathiane and 1,4-dithiane showed no effects on *D. magna*, despite being toxic for *A. fischeri* (Fig. 5).

We found that maximum solubility of 1,4-dithiane in water was much lower than reported in the literature (Table 1). We were not able to obtain in our experiments solutions of this compound that would exceed a concentration of 28 745 ± 2328 mg × L^{-1}, which is approx. 100x lower when compared with solubility value provided by Munro et al. (1999). The discrepancy may partly result from the fact that we used natural lake water, in which some salts have been dissolved. We have not observed, however, that solubility of the other compounds tested in our study was affected by the fact that we used lake water for preparation of the solutions.

The behaviour of daphnids exposed to HD (Fig. 3) seems to be unique and it is difficult to interpret. Initial immobilization immediately after contact with HD solution in the treatments with moderate and low HD concentrations is followed by return to active swimming by most animals, which coincides with the timing when most of HD is degraded (Fig. 4). Then after one day increased mortality can be observed. Similar observations of quiescence and return to active swimming was reported when daphnids were subjected to saxitoxin producing cyanobacteria (Bownik, 2017). This pattern raises the question regarding the mode of HD toxicity to crustaceans. Several non-exclusive hypotheses can be developed on the basis of our observations, e.g. chitinous carapace may be less permeable to HD than the skin of vertebrates, while moulting can replace damaged or contaminated carapace by a new, non-contaminated one. In this hypothetical scenario degradation products, some of which are even more toxic than HD, may be responsible for the mortality observed in the terminal phase of the experiment. Other hypotheses would be that HD acts as neurotoxic rather than blistering agent to crustaceans, or the increased locomotory response after initial immobilization (that may reflect shock driven by possible burns) is due to the tendency to escape from the contaminated/harming environment.

5. Conclusions

Results of the presented research provide a significant update of the aquatic acute toxicity threshold values of sulfur mustard and its degradation products. Despite its low water solubility and rapid hydrolyzation resulting in the 8 min 19 s long half-life (at 20 °C), when diluted, the sulfur mustard is toxic to *D. magna*. Moreover, two of its degradation products are more toxic than the parent compound. With the *D. magna* LC_{50} aquatic acute toxicity threshold at as low as 224 ± 12 μg × L^{-1}, 1,2,5-trithiepane is very toxic, being one of the most toxic CWA degradation products that have been investigated up to date. Observations of nonlinear immobilization effects of sulfur mustard exposure to *D. magna* in moderate concentrations requires more detailed data to verify newly formed hypotheses, making the mechanism of HD toxicity to crustaceans is worth further investigations. Since there are interspecific differences in susceptibility to the tested compounds, thus additional tests on other aquatic invertebrate and vertebrate species are necessary for assessment of ecological risks that HD and its degradation species may pose to aquatic ecosystems. Furthermore, for the sake of the quality of future risk assessments, more accurate data about CWA and their degradation products solubility is required.

CRediT authorship contribution statement

Michał Czub: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Jakub Nawała:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Stanisław Popiel:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing. **Daniel Dziedzic:** Data curation, Investigation, Validation, Writing - original draft. **Tomasz Brzeziński:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing. **Piotr Maszczyk:** Conceptualization, Data curation, Funding acquisition, Investigation, Supervision, Validation, Visualization, Writing - original draft. **Hans Sanderson:** Conceptualization, Data curation, Methodology, Software, Supervision, Validation, Visualization, Writing - review & editing. **Jacek Fabisiak:** Formal analysis, Funding acquisition, Resources, Supervision, Validation, Visualization, Writing - original draft. **Jacek Bełdowski:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - review & editing. **Lech Kotwicki:** Conceptualization, Data

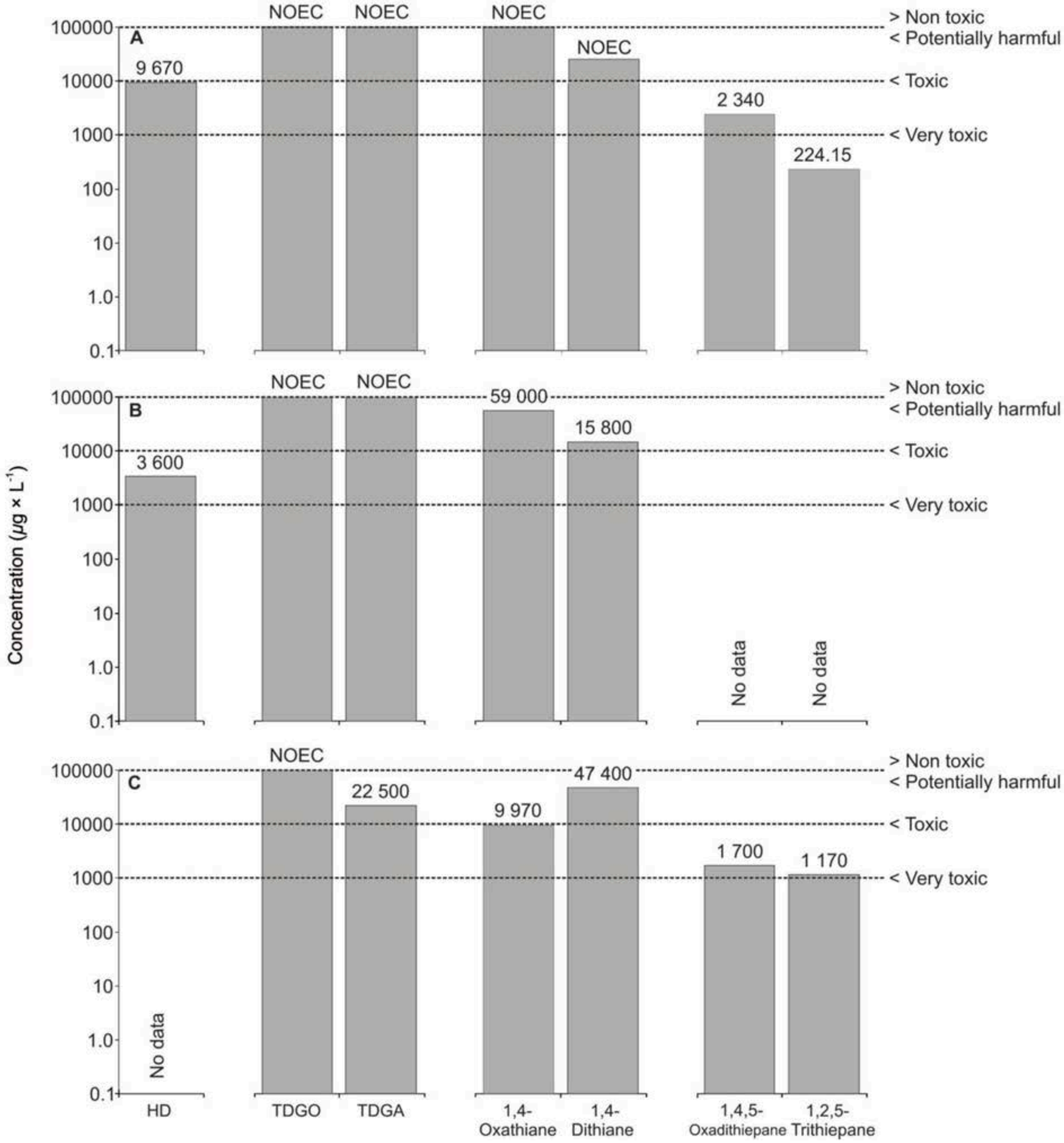


Fig. 5. Comparison of toxicity thresholds of sulfur mustard (HD) and its degradation products based on results from three different methods: A) Organization for Economic Co-operation and Development (OECD) Test No. 202: *Daphnia* sp. Acute Immobilization Test; B) Ecological Structure Activity Relationships (ECOSAR) and C) Microtox™ (Christensen et al., 2016). NOEC stands for No Observable Effects Concentration, meaning the compound is non-toxic for D. magna as there were no observable effects in highest tested concentrations (100 mg × L^{-1}) during the 48 h of exposure. Toxicity thresholds are applied according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research Paper no. 3

The effects of chemical warfare agent Clark I on the life histories and stable isotopes composition of Daphnia magna.

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The effects of chemical warfare agent Clark I on the life histories and stable isotopes composition of *Daphnia magna*[☆]

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ABSTRACT

Chemical warfare agents (CWA) dumped worldwide in all types of aquatic reservoirs pose a potential environmental hazard. Leakage of CWAs from eroding containers at dumping sites had been observed, and their presence in the tissues of aquatic animals was confirmed. However, the ecological effects of CWA have not yet been studied. In standardized laboratory bioassays, we tested if sublethal concentration of Clark I, an arsenic based CWA, can affect life histories (somatic growth rate, fecundity, size at maturity), population growth rate and stable isotope signatures of a keystone crustacean grazer *Daphnia magna*. We found that the life histories and fitness of daphnids reared in the presence of Clark I differed from those reared in Clark-free conditions. The effects were observed when Clark I concentrations were no less than 5 $\mu g \times L^{-1}$. With increasing concentrations of the tested CWA, all of the tested parameters decreased linearly. The finding indicates that even sublethal concentrations of Clark I can affect crustacean populations, which should be taken into account when assessing the environmental risks of this particular CWA. We found intraspecific diversity in susceptibility to Clark I, with some clones being significantly less vulnerable than others. We also found that in the presence of Clark I, the ratio of heavy and light isotopes of nitrogen in the bodies of daphnids was affected – daphnids exhibited $\delta^{15}N$ enrichment with increasing concentrations of this CWA. The isotopic composition of carbon was not affected by the presence of Clark I. The nitrogen isotopic signature may be used as an indicator of stress in zooplankton exposed to the presence of toxic xenobiotics.

1. Introduction

Sea dumping of Chemical Warfare Agents (CWAs) was a common practice worldwide during the 20th century. After World War II, massive quantities of chemical warfare agents, which had been captured by the Allies, were sunk in the seas under the provisions of the Potsdam Conference on Disarmament. Only in the Baltic Sea and its catchment approximately 50,000 tons of chemical weapons were dumped, while in Skagerrak the estimated amount of dumped chemical weapons is 168,000 tons (HELCOM, 1994; CHEMSEA, 2014; Nawała et al., 2016). The dumping of CWAs in seas, which had been considered the easiest way to dispose chemicals prohibited by the Chemical Weapons Convention, continued until the 1980s (Czub et al., 2018). Dumped CWAs still remain a major threat to marine and freshwater environments and to human health (Szarejko and Namieśnik, 2009; Radke et al., 2014). It has been found that shells and casings stored in deep sites have decomposed to such an extent that their contents (CWAs) have started to leak into the adjacent water and sediments (Bełdowski et al., 2016; Czub et al., 2018). Human activities at the dumping sites, such as bottom trawling or construction of offshore infrastructures, may additionally contribute to the spreading of CWAs from dumping sites (Czub et al., 2018). However, the possible impact of dumped CWAs on aquatic ecosystems has not been properly assessed. Only recently, several projects (CHEMSEA, DAIMON) have been launched to evaluate the possible ecological risks associated with dumped

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chemical munitions. Even so, there are still gaps in the knowledge about the very basic ecotoxicological parameters, such as LC_{50}, for several CWAs (Munro et al., 1999; Sanderson et al., 2007), which limits the predictive power of models developed to quantify the impact of these chemicals on ecosystems. As a consequence, the ecological risk of these substances may be underestimated. In particular, little is known on the effects of organo-arsenic based CWAs on aquatic biota. Regardless of their putative direct toxicity, products of their decomposition may be a source of subsequent secondary pollution, particularly due to the release of inorganic arsenic, which is of high toxicity, and may present an indefinite timeline for contamination (Radke et al., 2014; Greenberg et al., 2016).

Several types of organo-arsenic based CWAs were dumped into seas and freshwaters. Of these, one of the most frequently reported is Clark I. Clark I (diphenyl arsine chloride, diphenylchloroarsine, DA – Fig. 1) is an arsenic based chemical warfare agent. It was used in combat during World War I. After the war, it remained in the chemical weapons arsenals of several countries, moreover it was produced in large quantities, since it was also utilized as a riot control agent (Radke et al., 2014). Human exposure to the aerosolized agent results in nausea, vomiting, diarrhea, and mental state changes (Radke et al., 2014). Excessive exposure may result in death (Holstege, 2010); the LC50 for humans is 2 mg × dm^{-3} (Marrs et al., 2007). At ordinary temperature and pressure, it is made up of colorless crystals, characterized by low solubility ad low degradability (Radke et al., 2014). In aqueous solutions, it slowly degrades into diphenylarsinous acid, which either dimerizes or oxidizes to diphenylarsinic acid (Fig. 1, Niemikoski et al., 2017).

Large quantities of Clark I have been dumped into aquatic reservoirs around the world – the estimated amounts in the Baltic Sea are ca. 700–1500 t (HELCOM, 1994; Sanderson et al., 2009). Stockpiles of different organo-arsenic agents, including Clark I, were dumped in the North Sea, White Sea, Barents Sea, Mediterranean Sea, in the Atlantic Ocean off the northeast coast, in the Pacific Ocean off the coasts of Alaska and California, in several locations off the shores of Japan. Arsenic-based CWAs were also discarded in freshwater ecosystems (lakes and rivers) in Japan and Russia (Radke et al., 2014).

The toxicity of Clark I to aquatic organisms has not been measured. The only available aquatic toxicity thresholds of organo-arsenic CWA were modelled in ECOSAR, based solely on the physical and chemical parameters of each compound (Sanderson et al., 2007). We found only recently from an acute toxicity test in preliminary experiments that the 48h LC_{50} of Clark I for crustacean *Daphnia magna* is 37 μg × L^{-1} (Czub et al., unpublished). Metabolic pathways and molecular mode of action of arsenic-based CWAs in aquatic invertebrates have not been investigated. It is recognized that in vertebrates these xenobiotics are involved in inhibition of pyruvate dehydrogenase, up-regulation of unfolded protein response signalling, inflammatory responses and apoptosis (Li et al., 2016). Metabolised CWA-related phenylarsenic chemicals undergo conjugation with glutathione (GSH) (Niemikoski et al., 2020) and methylation, which may increase their toxic properties (Ochi et al., 2006).

Recent evidence confirms the continuous spread of Clark I and other CWAs from dumping sites (CHEMSEA, 2014; Bełdowski et al., 2017). An increase in As(III) concentrations in the Baltic, and the shoaling of its chemocline, have already been reported, and some authors hypothesize that this may be the result of the propagation of Clark I and its degradation products from dumping sites (Li et al., 2018). The presence of oxidized form of Clark I has been discovered in the tissues of fish and crustaceans sampled from populations living in areas adjacent to dumping sites (Niemikoski et al., 2017). This indicates that aquatic organisms in these areas can be exposed to the presence of non-lethal concentrations of this substance (and other CWAs released from dumped containers), and that it can accumulate in their bodies. However, it is not known whether, and to what extent, non-lethal concentrations of Clark I may affect the life-history traits of aquatic organisms. Life-history parameters, such as fecundity, size at first reproduction, somatic growth rate, and intrinsic population growth rate are crucial components of the fitness of zooplankton individuals, influencing different aspects of their ecology and evolution (Maszczyk and Brzeziński, 2018; Glazier, 2018; Gliwicz, 2003). The intrinsic population growth rate and somatic growth rate of cladocerans are frequently used as fitness surrogates (Lampert and Trubetskova, 1996).

Anthropogenic environmental pollution exerts pressure on the life histories of individuals and population characteristics (Yang et al., 2011; Arzate-Cárdenas and Martínez-Jerónimo 2012). This in turn affects micro-evolutionary processes, changes the community structure and ecosystem functioning (Medina et al., 2007;

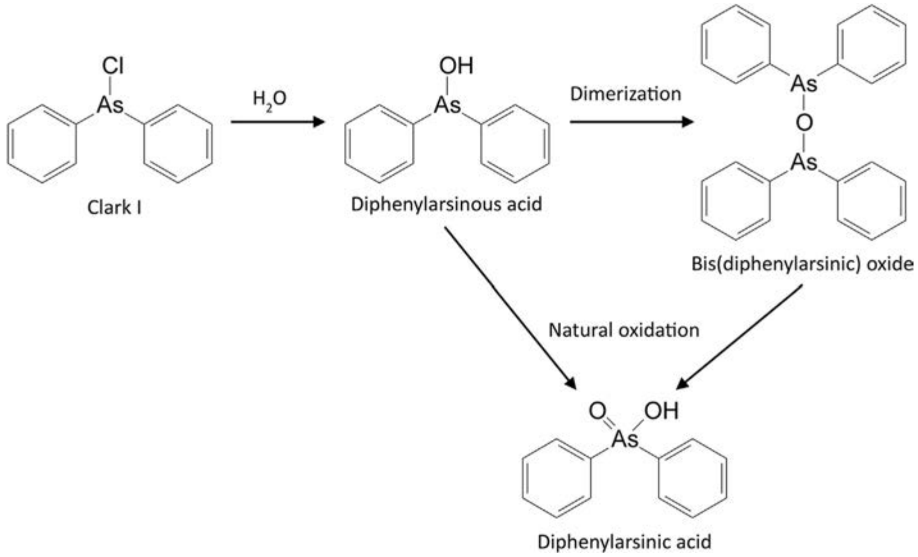

Fig. 1. Chemical structure of Clark I and its degradation products.

Giller et al., 2004). The non-lethal effects of toxic chemicals are, from an ecological and evolutionary perspective, as important as the direct lethal effects (Baird et al., 1990; Medina et al., 2007). Thus, **the first aim of our study was to test if sublethal concentrations of Clark I affect the life histories of the crustacean *D. magna***, and **if the effects of Clark I on the life histories of *D. magna* are concentration dependent**. *D. magna* is a model species in ecological and ecotoxicological studies, inhabits pelagial of different types of brackish and freshwater waterbodies, where it constitutes a key-stone species (Lampert, 2006; Lampert, 2011).

In order to evaluate the impact of pollutants on natural populations and to monitor the conditions of the organisms' development, a set of fast, reliable, and easy-to-use markers is required, which would allow for a rapid assessment of the effects on populations and provide a diagnostic/prognostic tool for decision making. Several methods have been developed, e.g. assesment of oxidative stress biomarkers (Wang et al., 2016; Wang et al., 2015) or life history parameters (Yang et al., 2011), see for review: Wernersson et al. (2017), Sarkar et al. (2006). However, the progress of new technologies allows new methods to be developed, which may be of potential use in tracing the condition of populations. One such method is the stable isotope analysis (SIA). It is widely used in paleontology and ecology as a tool for investigating climate change, reconstructing diet and trophic relationships (Gannes et al., 1998; Grey, 2006). Due to a reduction of the costs of the analysis, the number of studies utilizing this method is increasing. Yet, the explained variation of isotopic signatures (IS) of animals in nature and understanding the reasons influencing the IS is far from complete (Boecklen et al., 2011; Grey, 2006). For example, it was reported that fish living in wastewater polluted habitats showed ^{15}N enrichment (Schlacher et al., 2005); however, it was not explained whether this observation implies causality, nor what the mechanism behind it is. Nevertheless the stress elicited in organism by exposure to toxic compounds alter efficiency of energy acquisition, modify metabolism and induce compensatory mechanisms which further alter energy allocation (Baird et al., 1990; Bossuyt and Janssen, 2004; Wang et al., 2016), thus one may expect that such changes will affect fractionation of isotopes, which in turn will be reflected in changes of isotopic signatures of intoxicated organism. We have found no studies investigating the effects of pollution on the stable isotopic signatures of zooplankton. Thus, **the second aim of this study was to test if the composition of stable isotopes of carbon and nitrogen in *D. magna* are affected in the presence of toxic CWA.** We expected that the isotopic signatures of distressed daphnids would differ from those unexposed to toxic xenobiotics, and show enrichment with heavier isotopes. If so, isotopic signatures of organisms could be possibly used as an ecological indicator of environmental pollution, or more specifically – an indicator of distress caused by exposure of organisms to, at least some of, toxic chemicals.

2. Materials and methods

We determined somatic growth rates, population growth rates, fecundity and size at first reproduction of three clones of *D. magna* Straus, 1820, raised in the presence of different concentrations of Clark I. The clones are maintained at the facilities of the UW Department of Hydrobiology. The first clone (further referred to as DMB) originated from Grosser Binnensee (Germany), a brackish lake with an intrusion of saltwater from the Baltic Sea, the second (DMK) – from a temporary pond in Warsaw (Poland), the third (DMN) – from Novy Rvensky Rybnik, a permanent pond (Czech Republic). There are no dumping sites of CWAs in the vicinity of these locations. However, it could not be excluded that population from Grosser Binnensee was exposed to the possible presence of CWAs originating from intrusion of Baltic seawater. The ancestors of the tested individuals during the maintenance in the lab were not exposed to CWAs. The animals were cultivated in ADaM – an artificial medium for zooplankton (Klüttgen et al., 1994).

The food source was *Chlamydomonas rheinhardtii* (strain SAG 56) from a stationary phase, chemostat culture grown in WC medium (Guillard, 1975). The carbon content of the algal suspension was determined by reference to photometric light extinction at 800 nm, along with carbon-extinction equations determined previously using Multi N/C® 3100 analyzer from Analytik Jena AG (Germany).

Clark I (diphenyl arsine chloride) was synthesized by the Institute of Chemistry of the Military University of Technology (Warsaw, Poland), and its purity (98.6%) was verified using gas chromatography with mass spectrometry. Solutions of Clark I were prepared using ADaM medium. Four concentrations of Clark I were tested: 1.25, 2.5, 5, and 10 $\mu g \times L^{-1}$. The highest of the tested concentrations was approx. four times lower than the 48 h LC_{50} for *D. magna* (37 $\mu g \times L^{-1}$ as determined by Czub et al., unpublished). A fifth treatment (control) was free of Clark I. Temperature, oxygen concentration, pH, and conductivity in each treatment were checked using an Exo-1 YSI sonde.

Concentrations of Clark I in experimental media were measured in freshly prepared medium and in old medium that remained after the transfer of the animals to new beakers, using gas chromatography (Agilent Technologies 7890A) coupled with tandem mass spectrometry (7000 Agilent Technologies). Samples (30 mL) were transferred into a falcon tube where 1 mL of 1M hydrochloric acid (HCl) and 4 mL of methylene chloride with 5% (v/v) of 1-propanethiol were added. After 30 min of shaking tube was centrifuged at 2500 rpm for 10 min. 3 ml of the organic layer was transferred to a clean falcon tube and was dried with anhydrous $MgSO_4$. After 30 min of shaking the tube was centrifuged for 5 min at 2000 rpm. 1.5 mL of the supernatant was then transferred to chromatographic vials and the sample was analysed by GC-MS/MS.

The stable isotopic composition of carbon and nitrogen was determined using the Thermo Flash EA1112HT elemental analyzer coupled to a Thermo Delta V Advantage isotope ratio mass spectrometer. Isotope ratios are reported as delta δ and expressed relative to Vienna-Pee Dee Belemnite (VPDB) for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$. δ was normalized by a calibration curve based on international standards. Measurements were performed within an accuracy of ±0.12‰ for $\delta^{13}C$ and ±0.27‰ for $\delta^{15}N$.

The experiments were conducted in beakers filled with 0.1 L of medium, with a food level of 1.5 $mg \times C\ L^{-1}$. Every day fresh solutions of Clark I were prepared, and the medium in the beakers was renewed daily. Each treatment consisted of four replicates (beakers) per clone DMN and DMK and three replicates (beakers) per clone DMB. Since clone DMB is frequently used in experiments in our lab and its characterized by low variation in responses to environmental factors, we could reduce the number of replicates to three, thus reducing the number of animals suffering and killed during the experimental procedure. Two animals were kept in each replicate. Each clone was tested independently – in total 110 animals were assayed. All experiments were run at a constant temperature of 21 (±0.1) °C. The light conditions simulated the summer photoperiod (16:8 D:N). Light intensity, measured using a Li-Cor 189 quantum sensor (Li-Cor Biosciences®), was 0.32 ± 0.04 $\mu mol \times m^{-2} \times s^{-1}$.

The experimental animals originated from the third broods of mothers raised under non-limiting concentrations of *Ch. reinhardtii* for three generations. Synchronized cohorts of 12–24 h-old neonates were used to start the growth experiments. Initial weight was measured in two subsamples of 10 individuals. Growth experiments were run until the animals released the first clutch to the brood chamber, which occurred on fifth day of their lives. Eggs in the brood chambers were counted under a dissecting microscope, the length of the animals (size at first reproduction, SFR) was measured from the base of the tail spine to the upper margin of the compound eye to the nearest 0.01 mm. The animals were then transferred to preweighed tin boats (one boat per beaker), dried at 60 °C for 48 h, cooled in a desiccator, and weighed on an Orion Cahn C-35 electro-balance to the nearest 0.1 μg. Somatic growth rates were calculated from dry body mass as $g = (\ln M_t - \ln M_0) \times t^{-1}$, where M_t is the body mass at the end of experiment, M_0 – initial body mass of individuals and t is the time of maturation. The intrinsic rate of population increase was calculated in accordance with the Euler Lotka equation $r = \sum l_x m_x e^{-rx}$, where l_x represents age-specific survivorship, m_x – the number of newborns on day x, and x the age in days. Since there was no mortality in the experiment until the first clutch, l_x was set at 1 for this period. Since nonviable eggs were not found, the value of fecundity (number of eggs in the first clutch) was substituted by m_x under the assumption that all eggs would develop into alive animals.

For each clone, the relative change (sensitivity S_x) of each of the tested variables was calculated using the formula $S_x = ((X_{10} - X_0) \times X_0^{-1}) \times 100\%$, where X_{10} is the value of the tested variable (the juvenile growth rate (g) or the intrinsic rate of population growth (r), or size at maturity, or fertility) in the highest ($10\ \mu g \times L^{-1}$) concentration of Clark I; X_0 is the value of the tested variable in the absence of Clark I. A more-negative value of S_x thus indicates greater sensitivity to the presence of Clark I in water.

A general linear model and general regression model were used, which included Clark I concentration (a five-level factor, further referred to as Conc.), and clone (random factor with three levels, Clone). One value of each parameter from each beaker (in case of SFR and fecundity averages of all animals in beaker) was used in the statistical analysis as a replicate. Non-parametric analysis of variance (Kruskal-Wallis ANOVA) and the Mann-Whitney *U* test were used for the analysis of values of the relative change (the sensitivity) of the tested parameters. A significance level of $\alpha < 0.05$ was applied to all statistical analyses. The analyses were performed using Statistica v.13 (Statsoft Inc.) software.

3. Results

The values of initial nominal and analytical concentrations of Clark I in experimental solutions correspond to each other, the errors of all spectrometry-based measurements ranged between 1.19 and 10.5% (Supplementary data, Table 1). The arsenic in the experimental solutions was present only as organic form (diphenylchloroarsine, diphenylarsinous acid and diphenylarsinic acid). Average carbon isotopes ratio in algae during the experiment was $\delta^{13}C = -14.9 \pm 0.4‰$, average nitrogen isotopes ratio in algae was $\delta^{15}N = 1.58 \pm 0.39‰$.

We found that in the presence of Clark I all of the tested parameters: size at first reproduction (SFR), fecundity, somatic growth rate (*g*) and intrinsic population growth rate (*r*) decreased (Table 1, effect "Conc."). The fecundity and somatic growth rate of daphnids reared in $5\ \mu g \times L^{-1}$ and $10\ \mu g \times L^{-1}$ of Clark I were significantly lower than those of daphnids not exposed to Clark I (post-hoc test: Tukey HSD $p < 0.05$ and $p < 0.003$, respectively), the differences were not significant in lower concentrations of Clark I (Tukey HSD $p > 0.1$). Size at first reproduction and intrinsic population growth rate of daphnids raised in $10\ \mu g \times L^{-1}$ of Clark I were significantly lower compared to those of daphnids not exposed to Clark I (Tukey HSD $p < 0.0002$). The differences were not statistically significant in lower concentrations of Clark I (Tukey HSD $p > 0.05$). The lack of a significant interaction of "Clone × Conc." (Table 1) indicates that clones did not differ from each other in the direction of the response to the presence of Clark I in the experimental medium.

We found that the values of all of the investigated parameters: size at first reproduction ($F_{1,\ 55} = 50$ $p < 0.000001$, $R^2 = 0.48$), fecundity ($F_{1,\ 55} = 13.8$ $p < 0.0004$, $R^2 = 0.20$), somatic growth rate ($F_{1,\ 55} = 92$ $p < 0.000001$, $R^2 = 0.62$) and intrinsic population growth rate ($F_{1,\ 55} = 29$ $p < 0.000002$, $R^2 = 0.34$) decreased with increasing concentrations of Clark I within the range of the concentrations tested (Fig. 2).

At the highest of the tested concentrations of Clark I, the size at first reproduction of daphnids decreased on average by 7%–52%, depending on clone, fecundity decreased by 6%–19%, *g* by 24%–32%, and *r* by 17%–35% (Fig. 3).

We found significant interclonal differences in sensitivity to sublethal ($10\ \mu g \times L^{-1}$) concentrations of Clark I (Fig. 3). The relative decrease of fecundity (Kruskal-Wallis ANOVA $H_{2,\ 49} = 16.1$, $p < 0.0003$) and size at first reproduction (Kruskal-Wallis ANOVA $H_{2,\ 49} = 12.4$, $p < 0.002$) of clone DMB was higher than those of the other two clones, while the relative decrease of its population growth rate (Kruskal-Wallis ANOVA $H_{2,\ 49} = 7.4$, $p < 0.015$) was higher than in clone DMK (Fig. 3). The sensitivity of the DMK and DMN clones did not differ from each other. We found no interclonal differences when the susceptibilities of somatic growth rates to Clark I were compared (Kruskal-Wallis ANOVA $H_{2,\ 49} = 2.6$, $p > 0.3$).

The ratio of light and heavy carbon isotopes in daphnia tissues was not affected by the presence of Clark I, and remained at the same level across all of the Clark I concentrations tested (GRM $\delta^{13}C = -0.002[\text{Concentration}] - 16.8$, $R^2 = 0.0007$ $F_{1,\ 55} = 0.04$, $p = 0.8$ Fig. 4a). We found a weak positive relationship between concentrations of Clark I in the medium and the $\delta^{15}N$ ratio of daphnids tissues. Daphnids exhibited significant $\delta^{15}N$ enrichment with increasing concentrations of Clark I (GRM $\delta^{15}N = 0.047[\text{Concentration}]+3.15$, $R^2 = 0.14$ $F_{1,\ 55} = 9.1$, $p < 0.004$ Fig. 4b).

4. Discussion

4.1. The effects of Clark I on life history parameters of daphnids

Here we show that sublethal concentrations of Clark I affect the life histories and population characteristics of filter-feeding cladocerans. We observed effects at concentrations as low as $5–10\ \mu g \times L^{-1}$. All of the investigated parameters decreased substantially in the presence of this CWA. With increasing concentrations of Clark I, the magnitude of the reactions increased.

Particularly important is the decrease in size at first reproduction and somatic growth rate. These two parameters are widely recognized as crucial for the fitness of zooplankton – larger and faster growing individuals are better competitors for food than small and slow-growing individuals (Gliwicz, 2003). Reductions in SFR and *g* make zooplankton species vulnerable to becoming outcompeted and replaced by other species. Moreover, a reduction of body size reduces the efficiency of resource acquisition (Gliwicz, 2003). This in turn limits the ability of this key-stone species to control blooms of phytoplankton (DeMott et al., 2001; Gliwicz, 2003). The effects on fecundity and *r* are also important. Family planning in *Daphnia* is an important way of coping with ambient conditions (Glazier, 2018; Gliwicz, 2003). *Daphnia* exposed to cyanobacterial toxins enhance allocation to reproduction (Zhou et al., 2020). We have not observed such a reaction in daphnids exposed to Clark I – instead the fecundity decreased in the two highest concentrations of this xenobiotic. This may indicate that the strategies of dealing with these two different toxic compounds are different. A reduced amount of energy which can be allocated to reproduction constrains family planning, and makes cladocerans

Table 1

Results of the general linear models analyzing the effects of Clark I on life history parameters (size at first reproduction - SFR, somatic growth rate – *g*, fecundity) and population growth rate - *r* of *D. magna*. "Clone" – refers to the effects of random factor with three levels, representing number of clones analysed, "Conc." – refers to the effects of a factor with five levels, representing number of concentrations of Clark I tested, "Clone × Conc." – refers to the interaction of the two factors. Error df = 42, sample size n = 55. Statistically significant effects are bolded.

	SFR				Fecundity				*g*				*r*			
Effect	*MS*	*F*	*df*	*p*	*MS*	*F*	*df*	*p*	*MS*	*F*	*df*	*p*	*MS*	*F*	*df*	*p*
Clone	**0.03**	**5.04**	**2**	**<0.038**	**12.5**	**5.97**	**2**	**<0.026**	**0.02**	**17.2**	**2**	**<0.001**	**0.02**	**8.38**	**2**	**<0.011**
Conc.	**0.06**	**11.8**	**4**	**<0.002**	**9.18**	**4.38**	**4**	**<0.035**	**0.04**	**35.8**	**4**	**<0.001**	**0.03**	**16.9**	**4**	**<0.001**
Clone × Conc.	0.01	1.74	8	0.118	2.10	1.12	8	<0.372	0.00	1.63	8	0.147	0.00	0.41	8	0.911

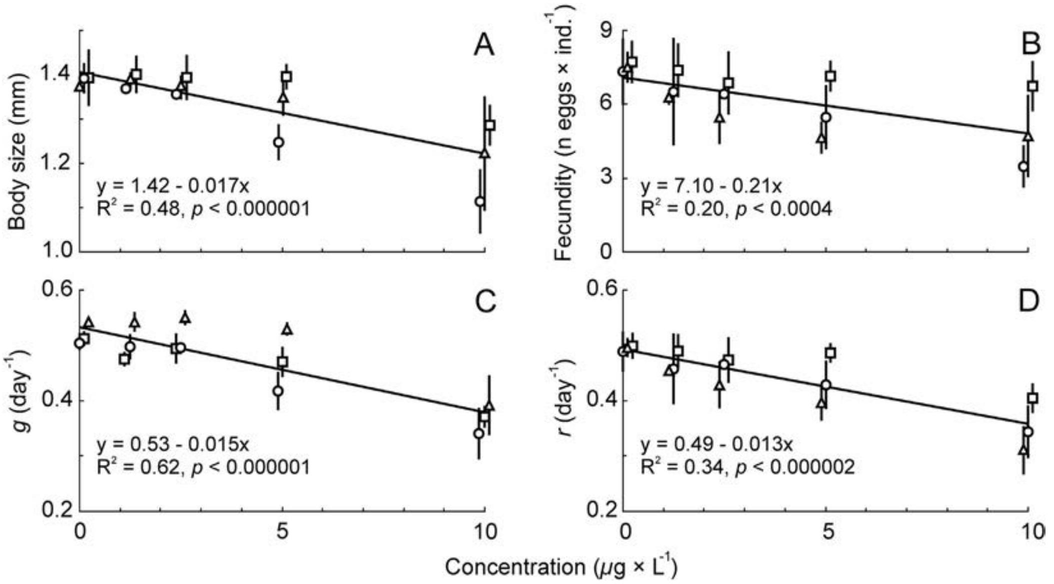

Fig. 2. Size at first reproduction (SFR) (A), fecundity (B), somatic growth rates (C) and. intrinsic population growth rates (D) of *Daphnia magna* (averages±SD of clone DMB – circles, DMK – squares, DMN – triangles) reared in different concentrations of Clark I. Lines of regressions calculated on the basis of replicates of all clones pooled together. Number of samples n = 55.

less able to cope with other environmental factors, such as predation. Adaptive response of cladocerans to increased fish predation involves increased fecundity and population growth rate (Gliwicz 2003). Since under toxic stress driven by Clark I fecundity and *r* is reduced, one may expect that the ability of daphnids to withstand fish predation under simultaneous pressure of both factors would be constrained.

4.2. Environmental concentrations of Clark I – are they high enough to affect local populations?

The estimated amount of Clark I that has been dumped in the Baltic Sea is ca. 700–1500 tonnes (HELCOM, 1994; Sanderson et al., 2009). If all dumped Clark I was dissolved in the total volume of Baltic waters, the resulting average concentration would be 0.05 μg × L^{-1}. This is two orders of magnitude lower that the lowest concentration, which in our study significantly affected the life-history parameters of the tested cladoceran species. Thus, one may argue that the risk posed by Clark I dumped into the Baltic Sea is negligible. However, one should bear in mind that our study examined the effects of Clark I during a relatively short exposure period (5 days). The lowest concentration of Clark I affecting the life-history and population parameters of cladocerans under chronic exposure is not known. This still does not account for the possible bioaccumulation of Clark I in the tissues of filter feeding crustaceans and further propagation to the next generation in the yolk of their eggs. Moreover, due to the morphometric characteristics of the Baltic Sea and its hydrological regime, it is not likely that the chemical would be equally distributed in the whole volume of water (Czub et al., 2018). One may expect that in the hypolymnetic waters and in the proximities of the dumping sites, concentrations of Clark I could be within the range that exerts an effect on crustacean zooplankton. The presence of Clark I and its degradation products in the sediments at the dumping sites has already been confirmed – the detected concentrations varied from 13.2 to 898.8 μg × kg dry weight^{-1} and from 5 to 47 μg × L^{-1} in pore waters (CHEMSEA, 2014; Bełdowski et al., 2016; Bełdowski et al.,

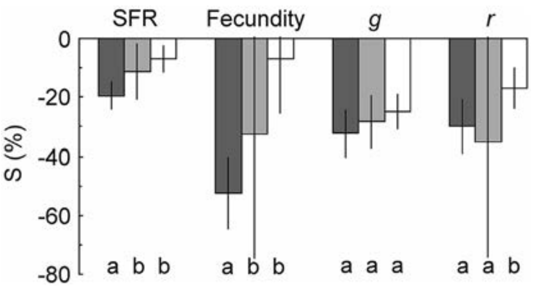

Fig. 3. Relative changes (the sensitivity) of size at first reproduction (SFR), fecundity, somatic growth rates and intrinsic population growth rates of three clones of *Daphnia magna* (white bars – clone DMK, grey bars – DMN, darkgrey bars – DMB). Sample size n = 49. Identical letters indicate homogeneous groups (U Mann-Whitney test, p > 0.05).

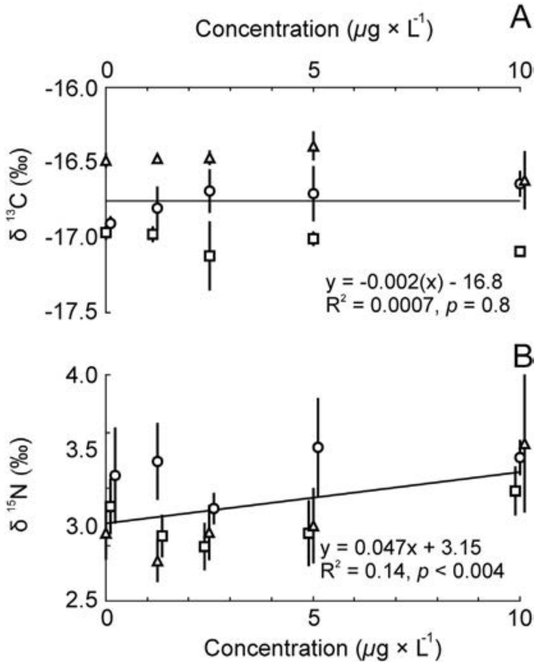

Fig. 4. Stable isotope compositions of carbon (δ ^{13}C - A) and nitrogen (δ ^{15}N – B) of *Daphnia magna* clones (circles represent averages±SD of clone DMB, squares – DMK, triangles – DMN) reared in different concentrations of Clark I. Lines of regressions calculated on the basis of replicates of all clones pooled together, sample size n = 55.

2017). In inland locations in freshwaters near dumping sites the concentrations reached 15 ppm (Ishizaki et al., 2005). These findings indicate that locally observed concentrations of Clark I and its degradation products are much higher than the concentrations identified in our study as causing sublethal effects in *Daphnia*. Moreover, one should bear in mind that in natural conditions organism is exposed to several stressors, which act simultaneously and may interactively enhance their negative effects (eg. Robinson et al., 2018). In our experiments daphnids were provided near optimal conditions, which can rarely be the case in nature. If so one may expect that the effects on life history in natural conditions can be observed in concentrations of Clark I lower than 5–10 $\mu g \times L^{-1}$. Identification of the interactive effects of different stressors acting together with this xenobiotic requires further studies.

4.3. Life-history parameters as indicators of distress driven by Clark I

Our findings indicate that sublethal concentrations of Clark I affect the parameters crucial for individual fitness and the population characteristics of zooplankton species, which should be taken into account when assessing the environmental risks of this particular CWA. Since *Daphnia* is a model species in ecotoxicological studies (Lampert, 2011; Tomasiks and Warren, 1996), our results provide a very basic canvas for ecological risk management, and a reference for estimating the toxicities of this xenobiotic for other species. All of the tested parameters can be used as bioindicators of physiological stress driven by Clark I. Decrease of somatic growth rate seems to be the most reproducible response among the three tested clones. Clark I is not the only organo-arsenic CWA, there are other such chemicals (lewisite, arsene oil, adamsite), which are even more toxic to humans than Clark I (Munro et al., 1999; Marrs et al., 2007). The impact of their ecotoxicological properties on aquatic organisms is not known, but one may expect that as with Clark I, they will also display a wide array of effects on aquatic organisms, even in sublethal concentrations. Our findings highlight the importance of incorporating the sublethal effects of CWAs into the models predicting the impact of these chemicals on aquatic biota.

4.4. The effects of Clark I on the isotopic signatures of daphnids

The need for development of tools to rapidly assess the effects of pollutants on individuals in natural populations is evident in the literature, reflecting the demand for reliable and effective ecological risk management. Different behavioural, physiological and molecular measures of toxicant-induced stress have been used and are still being sought as predictors of the effects of stress at the organismal level (Van der Oost et al., 2003; Sarkar et al., 2006). Recently, it was reported that fish living in wastewater polluted habitats showed significant ^{15}N enrichment (Schlacher et al., 2005; Conolly et al., 2013; Zhang et al., 2019), which may suggest that SIA could be used as an indicator of pollution. The authors speculated that this enrichment is due to fractionation of isotopes in inorganic pool of nitrogen in the environment, however they did not relate this to the condition of organisms living in polluted habitat. Our results show that indeed there is a relationship between the isotopic signature (δ^{15}N) of planctonic filter-feeders and pollution driven by the presence of a toxic arsenic-based chemical. With increasing concentrations of Clark I, the tissues of *Daphnia* became progressively enriched with heavier isotope of nitrogen. This effect, however, is not the result of differences in the elemental and isotopic composition of food, since daphnids in our experiments in all treatments were provided with food from the same source, of the same isotopic signature. We hypothesize that the observed effect reflects the general symptom of stress perceived by the animal. Pollution-driven stress affects the physiology of an organism. Daphnids exposed to toxic substances alter the allocation of energy among tissues and spend substantial amounts of energy on detoxification (Baird et al., 1990; Bossuyt and Janssen, 2004; Arzate-Cárdenas and Martínez-Jerónimo 2012). Faster tissue turnover promotes the fractionation of isotopes (Grey 2006). Contrary to the effects on nitrogen isotopic composition, the carbon isotopic composition was not affected by Clark I, which may indicate that only compounds and tissues rich in nitrogen undergo faster turnover. The increased metabolism of proteins and lowered level of lipid reserves may result in changes of isotopic signatures of an intoxicated organism, since a light isotope of nitrogen will be metabolised and removed from the organism faster than a heavy one.

4.5. Mechanisms responsible for observed changes in isotopic signatures

Arsenic tends to bind with sulfur-containing peptides, and formation of metallothioneins is a common way of getting rid of toxic organic-arsenic compounds (Shen et al., 2013), thus we expect that these peptides are up-regulated and metallothioneins are excreted when daphnids are exposed to the presence of Clark I. Glutathione-*S*-transferases (GSTs) and glutathione peroxidase (GPX) act also as detoxification system that counteracts oxidative stress in daphnids (Kim et al., 2010). Up-regulation of these enzymes and their increased activity may also contribute to enhanced metabolism of N-rich biomolecules and fractionation of ^{15}N. It requires further biochemical and physiological investigations to determine the physiological mechanism responsible for the pattern observed in our study. Determination of biochemical composition of animals reared in the presence of Clark I, preferably with an isotopic tracer, may unveil if indeed differences in body composition and tissue turnover occur between intoxicated and control animals. Changes in protein expression can be studied by transcriptomic/proteomic analysis of exposed and non-exposed individuals.

4.6. *Isotopic signature as potential indicator of distress driven by environmental pollution*

Although the relationship between the concentration of Clark I and the nitrogen isotopes ratio is statistically significant, its strength is not high, which may limit its applicability as an indicator of pollution-driven stress. This may reflect the fact of high interclonal differences in susceptibility to the presence of Clark I — clones more resistant to the toxicity driven by Clark I, with putatively less affected isotopic composition, may introduce variation, which diminish the effects observed at the level of species. However, if this is the case, this feature may be an advantage of using isotopic signatures as an indicator of distress driven by intoxication, which would allow to distinguish between populations on different stages of adaptation to the increased level of pollutant.

We suspect that the effect observed in our study is not specific for animals intoxicated by Clark I only, we expect that similar effects can be found in animals exposed to other toxic chemicals or unfavourable environmental conditions. It is likely that isotopic signature of animals could be used as a general indicator of stress, rather than an indicator of intoxication by particular chemical. This, however, requires further studies, examining wheter other pollutants (e.g. toxic heavy metals) or other environmental stressors (e.g. changes in pH, temperature, salinity etc.) may affect isotopic signatures of animals, and whether their impact on isotopic signaures is specific or non-specific.

It is commonly assumed that the isotopic composition reflects the diet of an individual („You are what you eat plus few per mil" paradigm (Gannes et al., 1998)). This is the foundation for using SIA for tracing trophic relations and paleoecological reconstructions. However, there is still unexplained variation in isotopic signatures of organisms, which may limit applicability of this method. According to our results the efficiency of fractionation of isotopes, which contributes to „few per mil", depends on the fact whether organism is exposed to toxic xenobiotic or not. Thus at least part of the variation of nitrogen isotopic signature can be explained by the consequences of distress driven by toxic xenobiotic. Our finding may help in interpretation of ecological reconstructions based on SIA.

4.7. *Interclonal differences in susceptibility to Clark I — ecological implications*

We found that within the tested species, significant differences exist in susceptibility to Clark I. Some clones of *D. magna* seem to be more vulnerable to Clark-mediated toxicity than others. This may indicate that physiological mechanisms exist in some clones that allow them to minimize the negative effects driven by Clark I on parameters crucial for individual fitness. Such intraspecific diversity would be of great importance for populations inhabiting areas threatened by the spread of Clark I. As a source for natural selection, this may trigger micro-evolutionary changes in populations. The selection of resistant genotypes may mitigate the negative effects of toxic chemicals on zooplankton populations, and result in increased tolerance to pollution (Medina et al., 2007). However, further investigations to properly assess changes in the genetic structure of populations due to Clark I pollution are necessary.

5. Conclusions

Sublethal, four-fold lower than the 48 h LC_{50}, concentrations of the organo-arsenic chemical warfare agent Clark I affect crucial life history and population parameters of a key-stone pelagic grazer *Daphnia magna*. With increasing concentrations of Clark I, the size at first reproduction, fecundity, somatic growth rate and population growth rate of daphnids decreased. The magnitude of the decrease of the observed parameters varied depending on clone, and reached up to 32—52%. There were substantial intraspecific differences with regard to susceptibility to Clark-mediated toxicity, with some clones being significantly less vulnerable than others. The presence of less vulnerable genotypes is a prerequisite for micro-evolutionary processes, which may allow populations to persist in polluted areas.

The daphnids exposed to the presence of Clark I exhibited an altered nitrogen isotopic composition — we observed the enrichment of the body with a heavier isotope as concentrations of this CWA increased. The isotopic composition of carbon was not affected by the presence of Clark I. The nitrogen isotopic signature may be used as an indicator of stress in zooplankton exposed to the presence of toxic xenobiotics.

CRediT authorship contribution statement

Tomasz Brzeziński: Supervision, Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Michał Czub:** Conceptualization, Investigation, Writing - review & editing. **Jakub Nawała:** Investigation, Writing - review & editing. **Diana Gordon:** Investigation, Writing - review & editing. **Daniel Dziedzic:** Investigation, Writing - review & editing. **Barbara Dawidziuk:** Investigation. **Stanisław Popiel:** Conceptualization, Methodology, Writing - review & editing. **Piotr Maszczyk:** Supervision, Conceptualization, Methodology, Investigation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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All applicable institutional and national guidelines for the care and use of animals were followed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115142.

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mój wkład polegał na:

koordynowaniu i nadzorze nad badaniami związanymi z syntezą bojowych środków trujących i analizą chromatograficzną próbek wody zawierających BST i produkty ich rozkładu, współpracy w opracowaniu wyników doświadczalnych; współudziale w przygotowaniu opisu części doświadczalnej; udziale w dyskusji otrzymanych wyników oraz pracach związanych z edycją wstępnego tekstu artykułu.

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